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ROLE OF CAVEOLIN-1 AND NRF2 IN NUTRITIONAL MODULATION OF PCB TOXICITY

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ROLE OF CAVEOLIN-1 AND NRF2 IN NUTRITIONAL MODULATION OF PCB TOXICITY

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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2015

ABSTRACT OF DISSERTATION

ROLE OF CAVEOLIN-1 AND NRF2 IN
NUTRITIONAL MODULATION OF PCB TOXICITY

Cardiovascular disease is the leading cause of mortality in Western societies and is linked to multiple modifiable risk factors including lifestyle choices. Emerging evidence implicates exposure to persistent environmental pollutants, such as polychlorinated biphenyls (PCBs), as a risk factor for the development or progression of cardiovascular disease. To reduce disease risks, it is critical to identify sensible means of biomedically reducing the toxicity of persistent organic pollutants and related environmental stressors.

First, we tested a hypothesis that endothelial cell inflammation and subsequent cardiovascular toxicity initiated by coplanar PCBs is modulated by the crosstalk between caveolae and Nuclear factor (erythroid-derived 2)-like 2(Nrf2) related proteins. Caveolae are lipid-enriched organelles found abundantly in endothelial cells and are important mediators of endocytosis and signal transduction. Caveolin-1 (Cav-1), the major structural protein of caveolae, is known to bind and concentrate multiple proteins related to cardiovascular disease and PCB toxicity. Downregulation of Cav-1 protects against PCB-induced vascular toxicity, but possible mechanisms of this defense remain elusive. Studies using endothelial cells isolated from mice deficient in Cav-1 as well as *in vitro* silencing assays demonstrated that loss of Cav-1 increases available antioxidant enzymes by upregulating the antioxidant master controller Nrf2.

Nutritional interventions focused on diets high in bioactive food components, such as polyphenols or certain fatty acids, may prove to be effective at decreasing environmental pollutant induced diseases. To test the hypothesis that dietary intervention can sensitize Nrf2 and/or caveolae signaling pathways, leading to a more effective anti-inflammatory defense against PCB insults, mice were fed a green tea polyphenol enriched diet and challenged with coplanar PCB 126. Mice fed an enriched diet and exposed to PCBs exhibited lower levels of oxidative stress and higher levels of multiple Nrf2 target antioxidant enzymes. Also, in separate *in vitro* studies, pretreatment of endothelial cells with the endogenously formed nutrient metabolite, nitro-linoleic acid, altered caveolae and Nrf2 related proteins, resulting in a modified response to PCB exposure. Together, these data support the paradigm that nutritional modulation may be a sensible means of reducing disease risks associated with exposure to environmental pollutants.

KEYWORDS: Cardiovascular Disease, PCBs, Nrf2, Caveolae, Nutrition

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April 13, 2015
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ROLE OF CAVEOLIN-1 AND NRF2 IN NUTRITIONAL MODULATION OF PCB TOXICITY

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Chapter one: Introduction

1.1 Background

1.1.1 Risk factors for atherosclerosis and cardiovascular disease progression

Cardiovascular disease (CVD) is the leading cause of death in the United States for both men and women and is associated with multiple modifiable and non-modifiable risk factors. CVD however is not a singular pathology but a group of disorders encompassing the heart, blood vessels, and associated tissues. Risk factors such as dyslipidemia, hypertension, obesity, sedentary lifestyle, and cigarette smoking have long been known to correlate with CVD, but recently nontraditional risk factors such as inflammatory status, insulin resistance, and genetic predisposition have emerged as additional important variables to identify and modulate in patients[1]. Although CVD is a broad and diverse pathology, there is evidence that an underlying condition called atherosclerosis may play a key role.

Atherosclerosis is a chronic inflammatory disease that can begin early on in life and eventually lead to blood vessel blockage and cardiac events such as stroke and myocardial infarction. Atherosclerosis is initiated when the lining of the blood vessel becomes damaged or activated by exogenous compounds such as lipophilic toxicants or by endogenous signaling mediators such as pro-inflammatory cytokines. This activation leads to increased blood flow, which aides in the recruitment of inflammatory blood cells such as leukocytes, as well as transcriptional upregeulation of adhesion molecules that bind and concentrate the leukocytes[2]. Upregulation of endothelial adhesion molecules, chemokines, and cytokines such as vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) in an NFκB mediated mechanism is critical for monocyte recruitment and infiltration (diapedesis) within the intima of the vessel[2]. Mature monocytes within an early atherosclerotic lesion, known as macrophages, can then upregulate receptors critical for the uptake of oxidized lipoproteins such as oxidized-LDL[3]. These lipid rich macrophages known as foam cells cluster together and can lead to fatty streaks and an atheromatous necrotic core[4]. As an atheroma progresses, nearby smooth muscle cells and macrophages undergo apoptosis which creates a “protective” fibrous cap[3]. In advanced stages of atherosclerosis, these caps may rupture and lead to a possibly deadly thrombotic event[3]. Although the hardening and thickening of vessel walls (atherosclerosis) requires multiple cell types, endothelial

cells appear to be a critical pathological mediator of both early and advanced stages of the disease.

1.1.2 Vascular endothelial cells- a link between nutrition, toxicology, and cardiovascular disease

Vascular endothelial cells are dynamic modulators of CVD and come in contact with heterogenous blood components and stimuli such as a wide assortment of dietary nutrients, lipid soluble toxic pollutants, and endogenous pro-inflammatory signals and compounds. Once believed to be a relatively unimportant regulatory cell type, endothelial cells are now known to be critical mediators of platelet adherence, modulators of vascular tone, regulators of thrombosis, and controllers of immune and inflammatory responses [5]. Endothelial cells display semi-permeable characteristics and can exert important paracrine and endocrine functions with surrounding smooth muscle cells and leukocytes[5]. Although endothelial cells create a relatively tight barrier between the blood and intima, controlled passage of proteins is possible via vesicular transport mechanisms such as lipid raft caveolae-assisted endocytosis[2]. Since endothelial cells are constantly exposed to blood and associated proteins, it is not surprising that nutrient and chemical compounds from the diet may play a critical role in the regulation of endothelial cell dysfunction and subsequent cardiovascular disease.

Dietary components may directly advantageously or detrimentally modulate endothelial cell function. A large body of evidence implicates certain nutritional choices as a means to protect against cardiovascular disease. For example, a highly studied class of nutrients known as omega 3 polyunsaturated fatty acids (PUFAs) may protect against atherosclerosis and cardiovascular disease by interactions with vascular endothelial cells[6]. PUFAs such as DHA and EPA found in fish oil may be protective for many reasons; they have been shown to decrease expression of endothelial cell adhesion molecules, increase protective nitric oxide formation from endothelial cells via endothelial cell nitric oxide synthase (eNOS), and reduce the levels of pro-inflammatory omega 6 unsaturated fatty acids in phospholipids[6]. Linoleic acid, the pro- inflammatory parent omega-6 fatty acid has been shown, through an NFκB mediated mechanism, to activate endothelial cells, upregulate Interleukin-6 cytokine production, release arachidonic acid, and allow for the leaking of albumin between endothelial cells[7]. The toxicity of linoleic acid deserves further investigation because it is a fatty acid found readily in Western diets and has grown in popularity since saturated and trans fat sources have been decreased due to highly publicized fears. Linoleic acid's pro-

inflammatory nature and endothelial cell toxicity may be attributed to the ability of the fatty acid to become oxidized and create detrimental metabolites such as 9- and 13 hydroxy-octadecadienoic acid (9- and 13-HODE)[8]. In addition to diet-derived fatty acids, endothelial cells can come into contact with multiple other bioactive food components, many of which can be described as polyphenols or flavonoids. For example the major bioactive component of green tea, epigallocatechin-3-gallate (EGCG) has been found at concentrations of 0.1- 1.0 μM in human plasma and has been shown to not only act as an antioxidant, but can directly bind to at least one specific receptor, (e.g., the laminin receptor) which is found on endothelial cells[9]. This paradigm that endothelial cells can directly sense and react to their ever changing plasma environment deserves much further investigation, but interestingly, the scope of this environmental sensing may not be limited to bioactive nutrients.

Accompanying nutrients from the diet, many lipophilic persistent organic pollutants (POPs) are also distributed throughout the body via blood flow and plasma proteins. Developing a body burden of POPs is not rare; for example, in studies attempting to identify the concentrations of multiple pollutant compounds in human plasma, it is not uncommon for the researchers to find known toxicants in 99-100% of their test subjects in the parts per billion range (ppb)[10]. Also, due to their lipophilic nature, many POPs such as polychlorinated biphenyls (PCBs) and other organochlorines can sequester within adipose tissue creating a large storage bank of highly concentrated toxicants which can be released in a steady state or acute time frame (e.g., weight loss)[11]. Not surprisingly, many of these manmade compounds have been shown to interact and activate endothelial cells; some causing toxicity via many of the same mechanisms as detrimental nutrient components. For example, certain toxicants such as PCBs have been shown to increase oxidative stress, upregulate NFκB, and lead to endothelial cell dysfunction and inflammation[12]. Due to commonalities between certain dietary nutrients and toxicants in regards to activation of endothelial cells, more research describing the interplay between nutrition and toxicology is critical to better understand the risks of environmental pollutants in a world of multiple concomitant stressors.

1.1.3 Polychlorinated biphenyls – an overview[13]

Polychlorinated biphenyls are a diverse class of manmade chemicals that have been outlawed in the United States since the 1970's, but still pose toxicological risks due to their persistence to environmental degradation[14]. Marketed for their effective thermal and electrical properties, PCBs became commonplace in capacitors, caulking, and hydraulic fluids but have become notorious for their correlations to multiple human toxicities ranging from endocrine disruption to vascular inflammation[15]. A major source of human exposure to PCBs is through dietary intake of contaminated foods and through inhalation of airborne pollutants[16]. Because most halogenated POPs, including PCBs, are lipid soluble, they easily accumulate in human tissues, leading to a perpetually increasing disease risk throughout a life span, especially in overweight populations[17]. PCBs are detrimental to multiple target organ systems and tissue types[16], but the breadth of this dissertation will focus on vascular related toxicity. Of the 209 individual congeners that were manufactured, coplanar PCBs, which lack chlorine substitutions on ortho positions of both phenyl rings, are most toxic to the endothelium and associated vasculature[18]. These non-ortho substituted PCBs such as PCB 77 and PCB 126 are able to, much like dioxin, bind to the aryl-hydrocarbon receptor (AhR) found within vascular endothelial cells and increase reactive oxygen species (ROS) through cytochrome P450 1A1 (CYP1A1)-mediated uncoupling[12b, 12c]. Mechanistically, an increase in cellular oxidative stress often precedes an inflammatory response[19]. CYP1A1 induction allows for the detoxification of multiple xenobiotics, but when in the presence of PCB, can become inefficient and leaky (i.e., uncoupled) and produce detrimental reactive oxygen species[20]. A hallmark of the pathology of vascular diseases, including atherosclerosis, includes a change in the cellular redox status and a resultant increase in oxidative stress, which favors chronic and low level inflammation[21]. There is sufficient evidence that POPs contribute to inflammation by activating oxidative stress-sensitive transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)[22]. For example, our previous studies suggest that PCBs, and in particular coplanar PCBs, can increase cellular oxidative stress and induce inflammatory parameters such as inflammatory cytokines, chemokines, and adhesion molecules in the vascular endothelium, which are metabolic events that foster an inflammatory response and atherosclerosis[12c, 22-23]. Through these pro-inflammatory mechanisms, PCBs and related environmental toxicants have been correlated with increased risk of multiple human chronic disease phenotypes including myocardial infarction, diabetes, stroke, and

hypertension[24]. Historically, epidemiological studies have been focused on occupational exposures such as those that occurred with Swedish capacitor workers in the mid-20th century, but it is becoming clearer that chronic low dose exposure may pose the most risk for the general public[25]. For example, populations most at risk for chronic exposure are those that ingest high levels of fatty fish due to the fact that PCBs preferentially bioaccumulate in adipose tissue and thus can be transported vertically through the food chain[24c, 26]. Although Inuit populations have been observed to have 3.4-fold higher plasma PCB levels than Caucasians, low ppb concentrations are common for the general United States population[27]. Importantly, according to National Health and Nutrition Examination Survey (NHANES) there appears to be an association with plasma levels of PCBs and cardiovascular disease in women in the United States[28]. Although these association studies are far from causative and can only correlate levels of PCBs with disease outcome, there are human studies that show PCB exposure can alter blood lipid profiles and increase total blood cholesterol and triglycerides, lending merit to the paradigm that environmental toxicants can promote or exacerbate vascular pathologies in humans[24c].

1.1.4 Epidemiological links between PCBs and cardiovascular disease

Cardiovascular disease (CVD) is a non-communicable disease that encompasses a group of disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism[29]. The World Health Organization estimates that 17.3 million people died from cardiovascular diseases (CVD) in 2008 alone, accounting for 30% of global deaths and serving as the number one cause of death globally[30]. Several risk factors and associated diseases including type 2 diabetes, hypertension, obesity, sedentary lifestyle, and over nutrition can contribute to the pathology of CVD. Furthermore, environmental pollutants, such as PCBs, can thus contribute to CVD directly or indirectly via promotion of these and other risk factors and associated diseases. Considering the significant burden that CVDs have on global mortality, it is imperative to explore and assess factors that may promote or exacerbate the pathogenesis of these diseases[30]. The goal of this section, then, is to summarize current epidemiological findings linking environmental exposures to PCBs with the development of CVD.

Type 2 diabetes

In 2000, an estimated 171 million people were diagnosed with diabetes; this estimate is expected to increase to 366 million by the year 2030[31]. Diabetes mellitus is characterized by poorly regulated high blood glucose levels and is primarily categorized into two groups, type 1 and type 2. Although most epidemiological studies do not distinguish between type 1 and type 2 diabetes, it is estimated that type 2 diabetes accounts for 90-95% of all diabetes cases[32]. Among patients with type 2 diabetes, CVD is the major cause of death, with more than 60% of patient deaths associated with myocardial infarction and stroke[33]. In addition, adults with diabetes have a 2 to 4-fold increased risk of CVD-related events compared to those without diabetes[34] and are at substantially higher risk for developing CVD[35].

Increasingly, data and an expanding body of literature are associating PCB exposure with heightened risk and incidence of type 2 diabetes development [36]. These studies involve a variety of cohorts and subject ages, suggesting that PCB exposure increase type 2 diabetes risk regardless of age or cohort. In addition to type 2 diabetes, PCBs also have been implicated in the development of gestational diabetes. Serum concentrations of PCB 138 and PCB 180 were associated with increased 2-hour glucose levels and PCB 180 with increased immunoreactive insulin levels, suggesting that PCBs contribute to insulin resistance in these mothers[37]. Gestational diabetes, characterized by the development of any degree of glucose intolerance in mothers during pregnancy, occurs during approximately 7% of pregnancies, or 200,000 cases annually. Women who develop gestational diabetes are at an increased risk for the development of type 2 diabetes after pregnancy[38].

While the molecular mechanisms that regulate the development of type 2 diabetes from PCB exposure remain elusive, several recent studies have shed light on this subject. Some, like the gestational diabetes paper, have proposed the development of insulin resistance as the mechanism by which PCBs cause type 2 diabetes[36d, 37, 39]. One recent study suggests that PCBs exhibit toxic effects by adversely affecting insulin producing β-cells in the pancreas as opposed to simply decreasing insulin sensitivity[40]. Additional studies have reported no association with exposure and insulin resistance[41], suggesting that the observed inverse relationship between PCB exposure and serum insulin[40] is indicative of β-cell toxicity. Impaired fasting glucose tests have also been reported to be associated with PCB exposure[42]. It has also been suggested that PCBs may modulate adiponectin levels, leading to increased rates of type 2 diabetes

development[43]. Adiponectin is a hormone produced by adipocytes that is significantly reduced in patients with insulin resistance, and is suggested as a strong predictor of type 2 diabetes[43a]. Studies have shown a negative correlation between plasma levels of adiponectin and plasma PCB 153 concentrations, which may explain the role of PCB exposure in the development of type 2 diabetes[43-44]. Additionally, PCB exposure has been associated with a decreased production of insulin growth factor I, providing another potential link between exposure to environmental pollutants such as PCBs and increased risk for the development of type 2 diabetes[45].

Hypertension

Of the 17.3 million deaths attributed to CVD in 2008, complications associated with hypertension, or high blood pressure, accounted for roughly half (9.4 million) of those deaths. Even more significant, these 2008 data indicated that 40% of all adults worldwide (aged 25 and above) had been diagnosed with hypertension, an increase from 600 million reported cases in 1980 to 1 billion cases in 2008. This drastic rise in rates of hypertension has been attributed to a number of factors including poor nutrition, lack of physical activity, and exposure to persistent chemical stressors[46].
Growing epidemiological evidence is substantiating a link between exposure to PCBs and increased risk of hypertension. The National Health and Nutrition Examination Survey (NHANES) data set has been a significant source of information on the association between hypertension and PCB exposure. Two recent studies showed that serum PCB levels were significantly associated with hypertension[47] and that serum PCB levels were, on average, higher among individuals with hypertension[47b]. Additional studies of NHANES data have indicated that dioxin-like PCB put a person at a higher risk for hypertension than non-dioxin-like PCBs, but that the arrangement of chlorines may also be an important factor[36c, 48].

While the NHANES data set has provided a wealth of information, a variety of epidemiological studies involving different cohorts have also found similar associations between PCB exposure and an increased risk of hypertension. Two studies of the Anniston, Alabama cohort, a group of 758 participants residing near the original Monsanto Corporation PCB manufacturing site, showed a correlation between rates of hypertension and serum PCB concentration[49], and that other than age, total serum PCBs were the strongest determinant of blood pressure level in 394 participants[50]

Additionally, a study of 1,374 Japanese residents substantiated these findings with their own data indicating that serum levels of dioxin-like PCBs are directly correlated with high blood pressure[51]. Finally, hospital discharge rates for hypertension for residents living in upstate New York in zip codes with POP contaminated sites were increased by 19.2%, although it is worth noting that these POP sites contained PCBs in addition to other pollutants[52].

Studies analyzing the correlation between PCB exposure and rates of hypertension have been performed with sample sizes from 59 participants to 12,200[47a, 50, 53]. With the exception of one, all studies found a significant relationship. Studies with sample sizes of 394 participants and above have shown a positive relationship between PCB serum level and rate of hypertension. An older study, with a significantly smaller sample of 59 participants failed to find a correlation[53a], suggesting sample size, as well as technological limitations, may have influenced the these findings.

Obesity

While atherosclerosis and hypertension are the most common risk factors for the development of cardiovascular disease, obesity serves as a primary modulator of these diseases and has been implicated heavily in CVD development[54]. Limited information has implicated PCB exposure in the etiology of obesity development, with primary correlations drawn from laboratory studies[14, 55] as well as from multiple epidemiological studies that have shown an association that warrants further examination[56].

Epidemiological studies into the association of PCB exposure and the development of obesity primarily have relied upon the comparison of serum PCB levels and body mass index (BMI) or birth weight, although studies have yielded mixed results. An interesting prospective study of 12,313 non-obese participants in the Seguimiento Universidad de Navarra (SUN) cohort showed that after a median 8.1 years there were 621 new incidences of obesity among these participants, with a direct correlation seen between increased dietary PCB intake (estimated from an earlier study of dioxin-like PCB concentrations in food) and incidences of obesity[56a]. Additional studies of adolescent and adult patients have reported total PCB[56b, 57] and congener-specific[58] obesity modulation, while others have found no correlation[59] or even an inverse association[60], indicating the complexity of this question. These discrepancies, and especially data indicating an inverse relationship between obesity rates and level of PCB

exposure, are likely due to bioaccumulation of highly lipophilic PCBs in human and animal fat tissue[44, 61] leaving low and/or inconsistent levels of PCB remaining in serum samples for analysis. This suggests that serum PCB concentration measurement may not provide an accurate estimate of a subject's PCB exposure or body burden that contributes to obesity risk factors. Recent weight loss or gain may also significantly alter the measured concentration of PCBs, which would also impact findings. While there are still many questions about the role of PCBs in modulating obesity risk factors, growing epidemiological evidence along with in vitro and in vivo findings have implicated PCBs as a part of a much larger group of environmental pollutants that act as obesogens, or chemicals capable of inappropriately activating molecular pathways that induce adipocyte differentiation and lead to a predisposition to obesity through dysfunctional weight-control[62].

Dyslipidemia

Dyslipidemia refers to a wide array of lipid abnormalities that can be causative in the development of CVD. Efforts to prevent CVD have focused significant effort on addressing dyslipidemia because it is readily modifiable by lifestyle changes and drug therapies, most notably statins[63]. There is compelling evidence that decreasing total cholesterol (TC), triglycerides (TG), and low-density lipoproteins (LDL) can help markedly reduce a patient's risk of CVD[35, 64]. Similar to findings on the association between obesity and PCB exposure, the most compelling and complete evidence comes from laboratory studies[65]. For instance, ApoE (-/-) mice injected with PCB 77 exhibited increased serum cholesterol and atherosclerosis[14]. Although the small volume of epidemiological literature directly examining a potential causal relationship between dyslipidemia and PCB exposure is certainly a limiting factor, serum lipid measurements are widely published within much larger analyses that implicate environmental stressors more broadly in metabolic dysfunction. Despite these limitations, though, there is epidemiological evidence suggesting PCB exposure may contribute to dyslipidemia[24c, 39, 51]. Among the contributors to dyslipidemia, elevated TG levels has been most consistently associated with PCB exposure[42, 66]. Increases in both LDL[67] and total cholesterol[53b, 67a, 68] have also been reported, although there are findings of no correlation[66b]. An inverse relationship between PCB exposure and high-density

lipoproteins (HDL) has also been shown[66c, 67b] which is significant considering that increasing levels of HDL are associated with a decrease in cardiovascular disease risk[69]

1.1.5 Mechanisms of coplanar PCB-induced vascular inflammation – a focus on the Aryl hydrocarbon receptor

Coplanar PCBs activate the AhR and cause increased oxidative stress and inflammation

The toxicity of coplanar PCBs, such as PCBs 77 and 126, is primarily induced through constant basal activation of the aryl hydrocarbon receptor (AhR), a transcription factor involved in xenobiotic metabolism. As a so-called orphan receptor, AhR has no associated high-affinity endogenous ligands, but rather binds to a wide array of ligands that include PCBs[70]. Without a ligand present, AhR exists in the cytoplasm of cells as an inactive complex with a heat-shock protein (Hsp90) as well as other co-chaperone proteins such as the p23 protein[71]. Once bound to a ligand, AhR translocates into the nucleus, dissociates from both Hsp90s and its co-chaperone proteins, and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR-ARNT complex then binds to a xenobiotic responsive element (XRE) in the promoter region of the target genes[72]. A large number of drug-metabolizing enzymes are induced as a result of AhR activation, including the phase I (oxidation), phase II (conjugation), and transporters of the phase III (excretion) metabolic pathways. The activation of these enzymes results in AhR ligands inducing their own metabolism and clearance from the body[70]. This, combined with the fact that AhR is a relatively ubiquitous protein that is expressed in most bodily tissues, has resulted in AhR being recognized as the body's primary molecular defense when presented with an environmental toxin[73]. Previous work has demonstrated that coplanar PCBs are capable of acting as AhR agonists[12b, 22, 73]. The toxicity that is then caused by these coplanar PCBs while stimulating AhR is often attributed to the release of reactive oxygen species (ROS) and the subsequent increase in oxidative stress[20]. It has been suggested that ROS-induced oxidative stress in conjunction with coplanar PCB exposure is the result of increased expression of, and eventual uncoupling of cytochrome P450 1A subfamily (CYP1A1)[20a, 22]. The metabolism of PCBs by cytochrome P450 enzymes involves a catalytic cycle of reactions that produce

metabolites of the parent compound[74]. The metabolism of PCBs is a slow process and, unlike many other drugs, the metabolites produced are not currently thought to be a major source of toxicity[20a]. The sluggish rate of PCB oxidation, though, causes the uncoupling of the CYP1A1 catalytic cycle and allows ROS to leak out of the active site, causing oxidative stress. Increased amounts of oxidative stress have been shown to be heavily involved in many of the risk factors for cardiovascular disease[75], including the development of atherosclerosis[76]. In addition to increasing oxidative stress, ROS also accelerate the degradation of nitric oxide, which impairs vasorelaxation by the endothelium and is often referred to as endothelial dysfunction[77]. Patients with coronary heart disease and increased endothelial dysfunction were shown to have an increased risk of cardiovascular events, demonstrating the importance of oxidative stress in cardiovascular disease[78].

In addition to increasing oxidative stress, coplanar PCBs acting as AhR agonists also induce cellular inflammation that is largely mediated by nuclear factor κB (NF-κB)[22, 79]. Previous research has shown that activation of NF-κB drives expression of target genes that activate immune response mechanisms, ultimately resulting in the release of proinflammatory cytokines and adhesion molecules that attract immune cells to modulate pollutant-induced toxicity[80]. These effects, combined with oxidative stress associated with ROS, have been shown to be atherogenic[81]. The compensatory mechanism intended to help combat these effects, known as the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant response pathway, regulates the response to oxidative stress and, once activated by ROS, Nrf2 protein binds to antioxidant response elements on target genes, leading to their upregulated expression. These target genes include xenobiotic metabolizing enzymes, such as glutathione S-transferases (GSTs), and many cytochrome P450s (CYPs), as well as antioxidant enzymes, NAD(P)H: quinone oxidoreductase-1 (NQO1)[82].

Role of AhR in cardiovascular disease

As seen in multiple epidemiological and laboratory studies, AhR ligands such as dioxins and coplanar PCBs can negatively affect the cardiovascular system and lead to phenotypes including hypertension and atherosclerosis[83]. Although the AhR's main evolutionary role may be to control detoxification and excretion of xenobiotic compounds, emerging evidence now shows a role for the AhR in endogenous

physiological functions. AhR is found in many cell types and tissues including placenta, lung, heart, and liver and can be activated by many non-toxicant ligands including arachidonic acid metabolites, heme metabolites, and LDL[84]. AhR knockout animals display developmental abnormalities, especially related to the vasculature. For example, deficient mice display aberrant hepatovascular and ocular blood flow due to improper developmental blood vessel formation[84b]. Xenobiotic and endogenous ligands may also promote aberrant blood pressure regulation through an AhR mediated mechanism. AhR null mice have been shown to have elevated angiotensin II and endothelin 1 levels which are known to be linked with hypertension, but these differences may also be related to specific environmental conditions such as geographic elevation[84a]. Interestingly, circulating AhR expression levels have been used as a surrogate biomarker for coronary arterial disease in certain populations, and specific AhR polymorphisms have been linked to a greater likelihood of developing the disease[85]. With an obvious role in proper mammalian development, early life exposures that alter AhR signaling may play a critical role in the toxicities of many environmental pollutants.

Cross-talk between AhR and other toxicant-related signaling proteins

AhR is known to cross-talk with multiple signaling partners, many of which are involved with inflammation and detoxification of xenobiotics. AhR has been shown to directly or indirectly alter the signaling of multiple proteins including hypoxia inducible factor (HIF), constitutive androstane receptor (CAR), estrogen receptor (ER), NFκB, caveolin-1, and Nrf2[12b, 86]. Although this may make deciphering the mechanisms of toxicity of environmental pollutants difficult, cross-talk between many of these pathways makes physiological sense. For example, many POPs including coplanar PCBs activate AhR and subsequent Phase 1 detoxification enzymes; while simultaneously activating Nrf2 and subsequent Phase 2 and 3 detoxification enzymes. Thus, AhR and Nrf2 pathways work concurrently to more effectively excrete and decrease the toxicity of pollutants. Mechanistically, the Nrf2 promotor contains AhR binding elements, whereas the AhR promotor contain Nrf2 binding elements[86b]. Also, certain compounds known as “bifunctional inducers”, such as β-napthoflavone, can activate AhR first, and only activate Nrf2 once CYP1A transforms the compound to a reactive intermediate[86b].

Cross-talk between AhR and NFκB is a complicated area of study, but one that deserves further investigation. Overall, it appears that NFκB suppresses AhR and AhR-

regulated CYP1A1 induction, but this is not universally agreed upon in the literature[87]. Directly, NFκB can act as a transcription factor and bind cis-acting sequences in the AhR promoter, leading to upregulation of CYP1A1 and related genes[87b]. Interestingly, NFκB has been shown to upregulate multiple cytochrome p450s directly. This upregulation has been shown to be due to multiple non-AhR mediated mechanisms including transcriptional and post-transcriptional means[87b]. However, it has also been shown that NFκB can directly suppress AhR-mediated pathways leading to exacerbated toxic responses in a preexisting inflammatory environment[87a]. This inhibitory cross-talk, is still controversial, as opposite effects are seen in certain cell types. Cell signaling pathways related to inflammation and xenobiotics are incredibly intertwined which makes it difficult if not futile to determine a direct mechanism of toxicant-induced disease. For example, CYP1A1 activation has long be studied and used as a surrogate/biomarker for AhR ligand binding. However, as more information related to the complicated cross-talk between these signaling pathways emerges, a larger view of mechanistic toxicology, including the roles of other AhR-related signaling pathways must be utilized to better understand all of the interactions.

1.1.6 Caveolae, Caveolin-1, and PCB-induced endothelial cell dysfunction

Caveolae are flask shaped organelles found primarily at the plasma membrane in endothelial cells, adipocytes, and other cell types[88]. Caveolae are composed of primarily cholesterol and sphingolipids, whereas the majority of plasma membrane is made up of phospholipids[89]. Caveolae have historically been believed to be primarily an endocytosis mediator, but emerging evidence implicates caveolae as a critical regulator of signaling transduction[90]. In fact, numerous proteins have been shown to be associated with/concentrated in caveolae membranes (see table 1.1). Important structural proteins of caveolae called caveolins (1, 2, or 3) are known to bind cholesterol, fatty acids, and many signal transduction related proteins[88-89, 91]. Caveolin-1 (Cav-1) has two important domains. The central domain contains 33 hydrophobic amino acids which act as a membrane anchor allowing for a hairpin structure that points both the N- and C-termini toward the cytoplasm[92]. The second important domain, known as the “Cav-1 binding domain”, allows for Cav-1 to bind itself as well as many other proteins[92]. Cav-1, a major component of endothelial cell caveolae, has been known to directly bind, and modulate many proteins related to inflammation, redox sensing, and cardiovascular

disease[88, 90, 93]. This has lead multiple research groups to examine the therapeutic potential of downregulating caveolin-1.

Due to their high abundance in cell and tissue types related to cardiovascular diseases, caveolae may have an important role in the pathogenesis of vascular disease. The creation of whole body knock out animals has allowed researchers to identify links between Cav-1 and caveolae to non-communicable diseases such as atherosclerosis and cancer[90, 93]. Currently, it appears that loss of Cav-1 may have protective or detrimental effects depending on what cell type, organ system, or disease pathology one is interested in[92-94]. Specifically relevant to this dissertation however, decreasing Cav-1 most likely is protective against the early stages of atherosclerosis and endothelial cell dysfunction[93-94].

Decreasing Cav-1 protects against atherosclerosis by multiple mechanisms. Over a decade ago it was determined that Cav-1 knockout animals had protection against atherosclerosis[94]. Early work showed that Cav-1-null mice bred with ApoE knockouts (spontaneously develop atherosclerosis) displayed a pro-atherogenic lipid profile, but interestingly showed dramatic decreases in lesion formation and decreased levels of pro-inflammatory adhesion molecules[94]. This protection may be due to decreased transcytosis of LDL particles across endothelial cells. In fact, some studies have shown upwards of 50% reduction in LDL uptake in cells lacking Cav-1[93]. Most of the anti-atherosclerotic phenotype may be due to loss of Cav-1 in endothelial cells[95]. A recent study clearly showed that overexpression of Cav-1 only in endothelial cells accelerated atherosclerosis as evidenced by increased levels of VCAM-1 in the vessel wall, increased atheroma formation, and decreased nitric oxide production[95]. Cav-1 has also been shown to regulate the expression of pro-inflammatory receptors such as TNF-α[96]. Taken together, a growing body of evidence now implicates a decrease in endothelial cell Cav-1 as anti-inflammatory and protective against the early stages of atherosclerosis. Previous work in our laboratory has also identified a role for Cav-1 and caveolae in PCB-induced endothelial cell toxicity.

Since PCBs are highly lipophilic we previously hypothesized a role relating lipid raft microdomain caveolae and associated proteins to PCB-induced vascular toxicity. Using cellular fractionation techniques and subsequently GC-MS to quantify PCB levels, we determined that PCBs selectively accumulate in caveolae[12b] and induce pro-inflammatory genes associated with caveolae. For example, we determined that PCB 77, a coplanar PCB, promotes phosphorylation of Cav-1 and subsequent

phosphorylation of eNOS, which when dysfunctional, can lead to the creation of reactive nitrogen species[97]. It is also known that AhR is critical for PCB-induced inflammation. Using an immunoprecipitation analysis, we determined that coplanar PCBs increase AhR binding to Cav-1 and that silencing of Cav-1(siRNA) attenuated PCB-mediated induction of CYP1A1, oxidative stress, and MCP-1[12b, 23b]. *In vivo,* we exposed ApoE-/- mice to coplanar PCB (49 mg/kg), and observed higher overall cholesterol levels, serum dyslipidemia and greater atherosclerotic lesion size. We demonstrated via real time PCR that PCB 77 treatment increased both aortic mRNA expression levels of MCP-1 in LDL-R$^{-/-}$ mice and plasma protein levels of MCP-1, suggesting that coplanar PCBs induce cellular dysfunction *in vivo*. However, no MCP-1 induction was detected in mice deficient in both LDL-R and Cav-1 genes[23b]. Similarly, Cav-1 KO mice did not induce the pro-inflammatory cytokines interleukin-6 (IL-6)[23b] when exposed to coplanar PCB. These findings imply that the loss of Cav-1 is protective of the earliest stages of PCB-induced inflammation and atherosclerosis. The mechanisms of this protection have remained elusive, however, interesting data has now linked loss of Cav-1 to an increase in protective antioxidant enzymes via an upregulated Nrf2 response[98]

1.1.7 The role of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in protecting against PCB-induced oxidative stress and vascular inflammation

Physiological systems have evolved multiple mechanisms of protection against xenobiotics and reactive oxygen species. Nrf2 has been shown to be a redox sensitive master regulator of antioxidant enzymes and plays an integral role in cellular protection against persistent environmental pollutants such as PCBs. There are multiple mechanisms of Nrf2 activation, including direct phosphorylation of Nrf2 by PKC delta and loss of contact between Nrf2 and inhibitory kelch-like ECH-associated protein 1 (Keap-1)[99]. Upon activation, Nrf2 is able to evade ubiquitination, enter the nucleus, and bind cis-acting antioxidant response elements (ARE) in target genes such as heme-oxygenase 1 (HO-1) and NAD(P)H:quinoneoxidoreductase 1(NQO1)[82a]. Importantly, Nrf2 activation leads to decreased inflammation which is a hallmark of PCB toxicity[100]. Primarily, activation of Nrf2 is regulated through interactions between Keap1. Modifications of Keap1 by oxidation or alkylation promotes a conformational change that eliminates an important Keap1:Nrf2 molecular connection and allows the protein lifespan

of Nrf2 to increase from 7-15 to 30-100 minutes[101]. Key residues in Keap1, such as Cys151, Cys273, and Cys288, control the interactions between Keap1, Nrf2, and ubiquitin ligase E3[101]. Genetic deletion of Nrf2 increases sensitivity to oxidants and inflammatory agents, whereas activation of the Keap1-Nrf2 pathway allows survival and adaptation under various conditions of cellular stress[82a, 102], suggesting that Nrf2 is a critical player in down-regulating pro-inflammatory genes[103]. Also, Nrf2 activation can play a role in atherosclerosis and vascular disease.

Atherosclerosis is a disease of inflammation and oxidative stress so it is not surprising that Nrf2 can play modulatory roles. In certain models of induced-disease, Nrf2-null mice display increased production of pro-inflammatory cytokines, chemokines, and adhesion molecules such as TNF-α, CXC, and VCAM-1[100]. Upregulating Nrf2, either via overexpression mechanisms or therapeutically, can decrease these pro-inflammatory mediators[100]. Activation of Nrf2 can lead to an upregulation of HO-1 which can suppress lesion formation by limiting the migration of monocytes into the intima[104]. HO-1 upregulation leads to an increase in endogenous antioxidants such as biliverdin and bilirubin which have been shown to decrease inflammation in many disease states. Interestingly, the endothelial cell response to reactive oxygen species (ROS) and reactive nitrogen species (RNS) may have a critical role in the development of atherosclerosis. As blood flows through vessels, a dragging force called fluid shear stress can activate enzymes such as NADPH oxidases resulting in the formation of superoxide[105]. Depending on the location of the vessel as well as other factors, endothelial cells may come into contact with disturbed flow or steady laminar flow. Disturbed shear stress (oscillatory/turbulent) has been shown to upregulate pro-inflammatory mediators such as MCP1 whereas laminar shear stress (steady) has been shown to be anti-atherogenic by upregulating Nrf2[105-106]. Endothelial cell activation of Nrf2 appears to be primarily anti-atherosclerotic, and our laboratory and others have also shown that Nrf2 is a critical mediator of toxicant-induced inflammation.

Genetic deletion of Nrf2 increases sensitivity to oxidants and inflammatory agents, whereas activation of the Keap1-Nrf2 pathway allows survival and adaptation under various conditions of cellular stress[82a, 102], suggesting that Nrf2 is a critical player in down-regulating pro-inflammatory genes[103]. Our data in ECs suggest a regulatory function of Nrf2 in PCB 126-mediated induction of CYP1A1, as silencing Nrf2 further increased CYP1A1 induction by PCB[73b]. Silencing of Nrf2 appears to shut off the

cellular defense against PCB toxicity as silencing of Nrf2 also increased inflammatory markers, such as MCP-1 and VCAM-1 at basal and post PCB-exposure conditions. Nrf2 has also been shown to protect against TCDD-induced oxidative injury and liver steatohepatitis, as Nrf2 deficient mice were less capable of preventing fat accumulation, lipid peroxidation, impaired adipogenesis, and depletion of glutathione when exposed to the toxicant[107]. Since Nrf2 is more of a redox sensing platform, a wide variety of activators have been identified and hypothesized including POPs, metals, nutrients, and chemotherapeutics. Many of these activators may be electrophiles or Michael reaction acceptors that can disrupt the Nrf2-Keap1 interactions[82a]. Interestingly, certain bioactive nutrients may be able to activate Nrf2 prior to a toxicological insult, which may help to prevent toxicant-induced disease.

1.1.8 Nutrition as a modulator of environmental pollutant-induced toxicity[13]

Sub-optimal or unhealthy nutrition modulates the toxicity of environmental pollutants

There is a large collection of evidence pointing to the role that a person's dietary makeup and eating habits can play in the promotion of chronic inflammation, metabolic disorders and vascular diseases[108]. Additionally, emerging data show that exposure to persistent organic pollutants such as PCBs may work in concert with unhealthy diets to promote cumulative or synergistic negative effects[109]. In fact, POPs and subprime nutritional status share mechanisms of disease development including the induction of pro-inflammatory pathways. Interestingly, a growing body of evidence implicates an exacerbated toxic effect of PCBs and other environmental pollutants when combined with an additive pro-inflammatory dietary environment[13b]. Our data support the paradigm that a poor nutritional state can exacerbate toxicity associated with exposure to PCBs and other persistent organic pollutants (POPs), and taking into account the interactions between diet and toxicants will help to better elucidate the impacts that PCBs and related compounds have on human health.

Multiple research groups have shown negative interactions between unhealthy or refined diets and environmental pollutants ranging from heavy metals to PCBs. For example, high fat diets appear to intensify arsenic-induced inflammation, hepatofibrogenesis, and cancer initiation[110], while diets high in saturated fats exacerbate

polycyclic aromatic hydrocarbon-induced adenomas[111]. Unfortunately, studies that investigate the impacts of mixing multiple types of exposures, (e.g. chlorinated pollutants and heavy metals) in combination with the added variable of an unhealthy diet are lacking. These types of comprehensive integrated toxicity studies will better mirror real world exposure conditions especially for people residing in close proximity to Superfund and other hazardous waste sites.

PCBs are prime pollutant candidates to study the interactions between diet and toxicants because they can sequester in adipose for long durations, and exposures are due often to ingestion of contaminated foods. Our research has focused on the interactions of PCBs and proinflammatory omega-6 fatty acids. We have shown that omega-6 fatty acids, and in particular linoleic acid, can cross-amplify the detrimental effects of coplanar PCBs and produce increases in oxidative stress, CYP1A1 induction, and endothelial permeability[109]. This work was expanded *in vivo* as we observed increased proinflammatory cytokine levels and lipid staining in mice fed oils rich in omega-6 fatty acids and exposed to PCB compared to mice exposed to PCB alone[112]. Animal fats such as those found in beef and chicken are significant sources of pro-inflammatory medium and long-chain omega-6 fatty acids as well as PCBs and other related POPs[113]. Mechanistically, synergism between unhealthy diets (e.g., high-fat/high caloric diets) and toxicants makes logical sense because both factors act upon many of the same receptors and cell signaling pathways. For example, omega-6 fatty acids and PCBs have both been implicated in inflammatory initiation through AhR, cytochrome P450's, and toll-like receptors (TLRs)[114]. With the growing rates of obesity and chronic oxidative and inflammatory stress, it is important to more thoroughly elucidate interactions between proinflammatory diets and toxicants. Although it does appear that unhealthy nutrition can work in concert with PCBs and related toxicants to create an increasingly proinflammatory phenotype, a new paradigm has emerged that implicates certain bioactive food components in protection against persistent organic pollutant-induced vascular toxicity.

Healthful nutrition decreases the toxicity of pro-inflammatory pollutants

Polychlorinated biphenyls and related Superfund POPs induce chronic oxidative stress and disregulated inflammatory responses, but anti-inflammatory nutritional antioxidants may buffer and protect against toxicant-induced disease through multiple cell signaling mechanisms[13b]. Polyphenols and bioactive fatty acids have been shown to decrease toxicant-induced maladies including liver diseases, tumor formation and growth, and endothelial cell activation[13b, 115]. Our work has shown that plant-derived flavonoids such as epigallocatechin-3-gallate (EGCG), and long-chain omega-3 fatty acids such as docosahexaenoic acid (DHA) can protect cellular systems by decreasing pro-inflammatory lipid raft signaling domains called caveolae and by simultaneously upregulating antioxidant defenses through increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation[116].

Polyphenols, for example, are an abundant and diverse class of bioactive compounds found in many fruits and vegetables and have been linked to decreased toxicity from dioxin and dioxin-like PCBs[117]. These ROS scavenging compounds can also increase PCB excretion rates[118], prevent AhR-induced inflammation[73b], limit body wasting[119] and decrease cellular dysfunction[120]. Resveratrol, a well-studied polyphenol, has been shown to interact with the primary receptor of coplanar PCBs, AhR, and to limit its activation and subsequent proinflammatory signaling cascade[121 122]. Other groups have shown that bioactive food compounds such as those found in broccoli (sulphoraphane) and red wine (resveratrol) can activate Nrf2 through multiple mechanisms and decrease oxidative stress levels in cells and in animals[123]. A goal of our research is to determine the efficacy of causative nutrients or plant-derived bioactive compounds found in everyday healthy diets, such as Vitamin E, that can bolster protective physiological mechanisms and prevent against PCB-induced vascular toxicity[115a]. Obviously, most Western diets are not fruit and vegetable focused, so it is important to identify and classify lipid-based bioactive compounds as well.

Omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the main components of fish oil and have been shown to decrease inflammation, reduce vascular diseases, and protect against dioxin and PCB-mediated toxicity[115b, 124]. Unfortunately, most Western diets lack adequate levels of omega-3 PUFAs, and the ratio of unhealthy omega-6 fats to omega-3 fats is very high[115b, 125]. Experimentally, it was determined that diets predominantly made up of oils rich in linoleic acid (omega-6) increased PCB-induced cellular dysfunction, but

this negative effect was blunted as the ratio favored protective omega-3's[126]. Interestingly, we have shown that mice fed a DHA-supplemented diet and subsequently exposed to coplanar PCB 126 exhibited a more profound antioxidant response as observed by higher expression levels of protective heme-oxygenase 1 (HO-1) and NAD(P)H:quinoneoxidoreductase 1 (NQO1)[127]. This preliminary work illustrates that omega-3 PUFAs may help to protect against PCB-induced vascular toxicity by allowing for a more efficient and intense endogenous protective response that utilizes multiple physiological cell signaling pathways. Polyunsaturated fatty acids, and especially omega-3 fatty acids, as well as their anti-inflammatory breakdown metabolites can protect against PCB-induced disease by activating Nrf2 but interestingly also by disrupting functional caveolae[116a]. Although caveolae and Nrf2 pathways appear to be prime candidates for modulation by bioactive food components, more research is necessary to better elucidate other novel pathways that can be targeted both in vitro and in vivo.

Healthful nutrition can decrease body burden of environmental toxicants

An emerging paradigm implicates healthful nutrition as an effective protective modulator of environmental toxicant-induced inflammation and human disease. Multiple laboratories have investigated the decreased toxicities of environmental pollutants due to bioactive nutrients such as flavonoids and omega-3 polyunsaturated fatty acids, but many of these studies rely on in vitro assays that lack the complexity of a whole body organismal approach. Importantly, emerging classes of bioactive food components such as polyphenols also have been shown to modulate the pro-inflammatory effects of environmental toxicants. Our laboratory and others have shown that a wide array of phenolic compounds such as EGCG, curcumin and quercetin can decrease toxicant-induced oxidative stress and inflammation in multiple cell types, tissues and animal species[118-119, 128]. Although human studies are lacking, strong evidence in animal models implicates a protective role for flavonoids and other polyphenols perhaps through the induction of antioxidant enzyme pathways and increased fecal excretion rates[129]. Interstingly, plant-derived polyphenols are being found to not only protect against POP-mediated oxidative stress, inflammation, and toxicity but also to bind to POPs and thus

contribute to a decrease in body burden. In fact, if has been proposed that green tea, containing high levels of polyphenols including EGCG, can inhibit the intestinal absorption of lipids and highly lipophilic organic compounds and accelerate excretion of PCBs[118, 130].

Certain foods and polluted air can be major sources of toxicant exposures[16]. Thus, altering nutritional choices may prove to effectively modulate risks associated with exposure to POPs. Making informed dietary decisions such as substituting lower fat versions of protein sources (e.g. legumes, nuts, or lean meats and dairies) may have multiple health benefits including decreased exposure to detrimental pollutants such as dioxins and PCBs[131]. For many populations it may be difficult or cost-prohibitive to change major dietary protein sources; thus, increasing dietary intake of other bioactive nutrients may buffer already exposed individuals against the negative ramifications of pollutant exposures. For example, several laboratories have shown that diets high in fiber can alter the absorption and excretion rates of pollutants such as PCBs[132]. Mechanistically, multiple dietary fibers have been shown to effectively bind pollutants such as dioxins, which may help to explain increased fecal excretion rates, decrease in body burden, and observed protection[132a]. Increasing excretion of lipophilic toxicants through other nutritional compounds such as fat substitutes may also effectively decrease risk. For example, much emphasis including human clinical trials has been placed on the interactions between the non-absorbable fat substitute olestra and environmental toxicants[133]. Researchers determined that low levels of olestra supplementation (25 grams/day) increased the excretion of multiple PCBs and related contaminants upwards of 11 fold compared to normal diet[134]. Interestingly, in a human study, an olestra-supplemented diet completely eliminated excessive POP concentrations in adipose tissue and reversed POP-mediated clinical conditions such as diabetes and hyperlipidemia[135]. Diet-derived bioactive compounds such as EGCG are attractive modulators of toxic exposure because they may both help to prime the physiological system prior to a toxic insult by upregulating protective detoxifying enzymes as well as protect therapeutically after exposure by increasing the rate of excretion and lowering overall body burden[129b]. Altering diets to increase fiber and bioactive nutrients from fruits and vegetables is an effective and cost-efficient means of modulating POP toxicity.

Protective cellular signaling pathways

Multiple signaling pathways have been attributed to nutritional modulation of environmental toxicants, but we have shown that caveolae and the antioxidant master controller nuclear factor (erythroid-derived 2)-like 2 (Nrf2) play integral protective roles. There is increasing evidence that lipid raft membrane microdomains, i.e. caveolae, may play an important role in atherosclerosis, the toxicity of coplanar PCBs, and nutritional protection[12a, 90, 94]. Caveolae are flask shaped membrane invaginations that are important in cholesterol transport, nutrient and xenobiotic import into cells and cellular signaling[93]. Due to the lipophilicity of PCBs, they may enter the cell through lipid raft mediated events or come into contact with caveolae-related signaling proteins. Caveolae are abundant in vascular endothelial cells and the cardiovascular system in general, which points to a highly probable role for caveolae in inflammation and atherosclerosis[92]. Our laboratory has previously reported that coplanar PCBs promote the upregulation of genes related to the activation of endothelial cells and the initial stages of atherosclerosis and that the loss of functional caveolae ameliorates these detrimental effects[97]. Caveolin-1 (Cav-1), the major structural protein of caveolae, contains the "Cav-1 binding domain" that is known to bind multiple proteins including endothelial nitric oxide-synthase (eNOS), v-src sarcoma (SRC), protein kinase C (PKC), extracellular-signal-regulated kinase(ERk) and protein kinase B (Akt); many of which are involved in inflammatory pathways[90]. For example, downregulation of Cav-1 can lead to eNOS activation and subsequent increases in diffusible nitric oxide, which has been shown to play a major role in healthy blood pressure and vessel tone[12c]. Many publications from our laboratory and others show downregulation and elimination of Cav-1 to be anti-atherosclerotic[12b, 23b]. Importantly, eliminating Cav-1 prevents PCB-induced cellular dysfunction[97]. Our data point to Cav-1 as a possible anti-atherosclerotic therapeutic target and we hypothesize that nutritional intervention can downregulate Cav-1 and, in turn, protect against PCB-induced inflammation.

Nrf2 is a transcription factor that can upregulate cytoprotective genes in response to oxidative stress, xenobiotics, and bioactive food molecules[100, 136]. Many nutrients, including resveratrol, sulforaphane, and epigallocatechin gallate (EGCG), have been shown to activate Nrf2[123c, 137]. There are multiple mechanisms of Nrf2 activation, including direct phosphorylation of Nrf2 by PKC delta and loss of contact between Nrf2 and inhibitory kelch-like ECH-associated protein 1 (Keap-1)[99]. Upon activation, Nrf2 is able to evade ubiquitination, enter the nucleus, and bind cis-acting antioxidant response

elements (ARE) in target genes such as heme-oxygenase 1 (HO-1) and NAD(P)H:quinoneoxidoreductase 1(NQO1)[82a]. Importantly, Nrf2 activation leads to decreased inflammation which is a hallmark of PCB toxicity[100]. Activation of Nrf2 may lead to vascular protection from PCB-induced toxicity and we hypothesize that a diet rich in bioactive food components can activate Nrf2 and prevent PCB-induced inflammation. Nrf2 has been shown to cross-talk with multiple signaling partners, especially with the major player in PCB toxicity, AhR[138]. Although it has been known for decades that dioxin and dioxin-like compounds activate both AhR- and Nrf2-related genes, it was shown only recently that Nrf2 is required for induction of AhR genes such as CYP1A1[139]. This observed cross-talk can be explained mechanistically at the genetic level by the fact that the promoter region for Nrf2 contains AhR binding regions and the gene promoter for AhR contains multiple Nrf2 binding elements[138]. Our laboratory previously determined that AhR is a binding partner of Cav-1, so it is plausible that cross-talk between caveolae and Nrf2 signaling pathways also exists[12b].

Little is known about the cross-talk between Nrf2 and caveolae signaling and how bioactive compounds such as omega-3 lipids, flavonoids and other polyphenols interact to protect against environmental insults. We have shown that decreasing cellular Cav-1 levels results in a more intense antioxidant response. Mechanistically, we attribute this to decreased AhR activity as well as increased Nrf2 activity[73b]. Recently, an intimate example of cross-talk between Cav-1 and Nrf2 was illustrated[140], and our published data supports this phenomenon[98, 116b]. Using bioactive food components such as flavonoids and omega-3 PUFAs may be an economically and logistically beneficial method to counteract PCB-induced vascular inflammation. Although it does appear that both caveolae and Nrf2 signaling pathways play an important role in nutritional modulation of vascular inflammation, other signaling cascades may also prove to be involved. Phytochemicals may work through multiple mechanisms that upregulate protective genes, downregulate pro-inflammatory genes and/or decrease overall oxidative stress (Figure1.1).

Emerging and future directions

The paradigm of nutritional modulation of environmental toxicants is still new, thus many issues remain unresolved. One critically important area in need of further investigation involves the metabolism of protective nutrients. With the advent of more

precise and high-resolution analytical techniques, researchers have finally begun to elucidate truly causative bioactive food components. In a physiological system, both nutrients and xenobiotics interact with similar cell signaling molecules and pathways. Both nutrients and toxicants are impacted by all aspects of absorption, distribution, metabolism and excretion, and understanding how bioactive compounds are altered or influenced at each step will allow for more efficient biomodulation. Specifically, the detection and identification of bioactive metabolites is of utmost importance due to the fact that many parent compounds are altered and modified in vivo. As mentioned previously, resveratrol is an extensively studied protective phytochemical, and exemplifies the necessity of further investigating the roles that bioactive metabolites play. For example, a major limitation with resveratrol supplementation involves poor gut absorption of the parent compound and fast metabolism to sulfate and glucuronide products[141]. However, even with the short half-life of the parent compound, there is a large body of evidence associating resveratrol supplementation with protective phenotypes *in vivo*[123a]. This has led some research groups to investigate the bioactivity of specific metabolites and observed protection via the activation of Sirtuin1(SIRT1) and inhibition of cyclooxygenase[123a]. This is an important finding worthy of further exploration because bioactive metabolites, such as the glucuronide and sulfate forms of resveratrol, have much longer half-lives than the parent compound, which may qualify the metabolites as more appropriate and effective nutritional modulators[142]. Also, our laboratory has shown that oxidized metabolites of the bioactive omega-3 PUFA DHA are more protective against such PCB-induced vascular toxicity compared to the parent PUFA[116a]. Mechanistically, oxidation of PUFAs can lead to multiple F-type isoprostanes with active cyclopentenone rings that have been shown to activate Nrf2, inhibit NFκB, and decrease inflammation[143]. Also, other metabolically-relevant electrophilic fatty acid modifications, such as nitro-fatty acids, may play a role in combating inflammation and environmental toxicant-induced diseases[144]. Nitro-fatty acids are a newly discovered class of modified lipids that exhibit anti-inflammatory properties via multiple mechanisms[145]. Unsubstituted fatty acids such as oleic acid (18:1) and arachidonic acid (20:4) can be nitrated endogenously by multiple enzymatic and non-enzymatic reactions, resulting in the inhibition of pro-inflammatory mediators, induction of protective heme oxygenase-1 and the relaxation of blood vessels[146]. This class of bioactive lipid can protect via multiple mechanisms including upregulation of PPARγ and Nrf2 as well as downregulation of pro-inflammatory NFκB[147]. More work needs to be accomplished to

elucidate the mechanistic impact of nitro and oxidized PUFAs on vascular inflammation and toxicant-induced diseases. It appears that both oxidized PUFAs and nitro-fatty acids are anti-inflammatory, which may implicate the importance of bioactive modified lipids in a clinical and therapeutic setting. Although research linking bioactive lipids, vascular inflammation and environmental toxicants is lacking, state of the art high-resolution mass spectroscopy technologies are beginning to allow researchers to explore the most efficient and physiologically-relevant bioactive compounds, which will in turn help further the use of nutrition in a clinical and preventative setting.

1.2 Scope of Dissertation

1.2.1 Aims of dissertation

The general hypothesis of the research described herein is that coplanar PCBs can promote cardiovascular disease by modulating endothelial cell caveolae. We also hypothesize that nutritional modulation of PCB-toxicity is modulated through the cross-talk between caveolae and Nrf2 signaling pathways. To test these hypotheses the following Specific Aims were proposed:

Specific Aim 1: To test the hypothesis that endothelial PCB toxicity and associated inflammatory events are regulated through the crosstalk between caveolae and Nrf2 related proteins such as Keap1.

Specific Aim 2: To test the hypothesis that dietary intervention with green tea polyphenols can sensitize Nrf2 and/or caveolae signaling pathways, leading to a more effective anti-inflammatory cellular defense/response against PCB insults *in vivo*.

Specific Aim 3: To test the hypothesis that treatment with nutrient metabolites, such as nitro-fatty acids, instead of parent compounds will better sensitize Nrf2, caveolae, and/or AhR signaling pathways leading to a more effective anti-inflammatory cellular response against PCB insults.

Table 1.1 Proteins related to inflammation and atherosclerosis that are concentrated in caveolae lipid micro-domains[88]

Protein Name	Function Related to Atherosclerosis
Cav-1	Inhibits eNOS, cholesterol regulation
SRC	Plaque destabilization
eNOS	Vascular tone, Superoxide production
ERK	Inflammation
AhR	NFκB induced inflammation
CD36	Involved in fatty acid uptake
PKC	Inflammation, Vasoconstriction
ER α and β	Control of eNOS
SR-B1	HDL scavenger receptor
gp60	Control of albumin transcytosis
Fyn	Negative regulator of Nrf2
MMP-1/2	Plaque destabilization

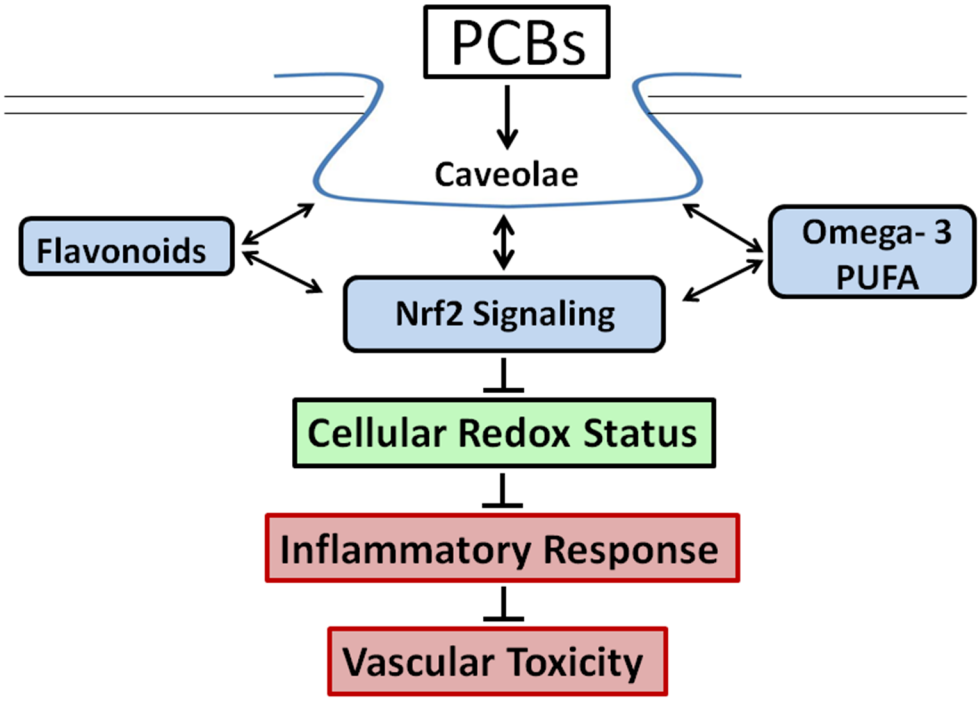

Fig. 1.1 Nutritional modulation of PCB-induced vascular toxicity. Bioactive nutrients such as certain flavonoids found in fruits and vegetables or bioactive fatty acids such as long chain PUFAs may work through caveolae and/or Nrf2 signaling to decrease the toxicity of PCBs and related persistent organic pollutants (POPs). Coplanar PCBs induce oxidative stress and inflammation, while certain bioactive food components are anti-inflammatory and may be able and decrease the severity of toxicant-induced disease.

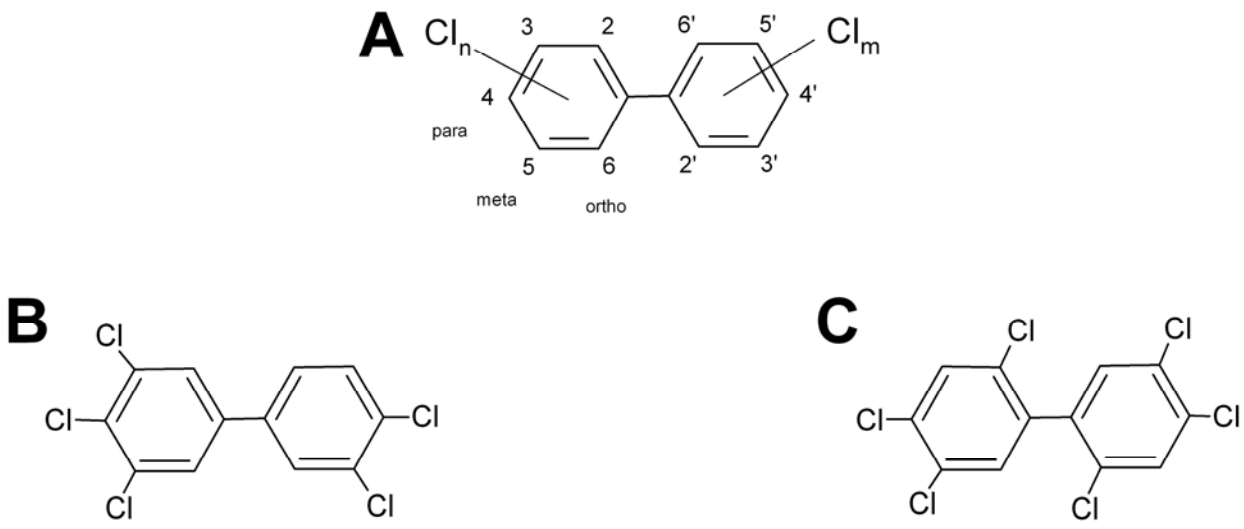

Figure 1.2 The configuration and amount of chlorines evident in a specific PCB congener relates directly to the molecular mechanism of toxicity of that congener. **A.** General structure of a polychlorinated biphenyl with relevant nomenclature highlighted **B.** Structure of 3,3',4,4',5-Pentachlorobiphenyl (PCB 126), a coplanar, non-*ortho* substituted PCB that exerts toxicity primarily through the AhR **C.** Structure of 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153), a non-coplanar, di-*ortho* substituted PCB that does not bind the AhR and exerts toxicity primarily through endocrine disruption.

Chapter 2: PCB 126 toxicity is modulated by cross-talk between caveolae and Nrf2 signaling[98]

2.1 Synopsis

Environmental toxicants such as polychlorinated biphenyls (PCBs) have been implicated in the promotion of multiple inflammatory disorders including cardiovascular disease, but information regarding mechanisms of toxicity and cross-talk between relevant cell signaling pathways is lacking. To examine the hypothesis that cross-talk between membrane domains called caveolae and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathways alter PCB-induced inflammation, caveolin-1 was silenced in vascular endothelial cells, resulting in a decreased PCB-induced inflammatory response. Cav-1 silencing (siRNA treatment) also increased levels of Nrf2-ARE transcriptional binding, resulting in higher mRNA levels of the antioxidant genes glutathione s-transferase and NADPH dehydrogenase quinone-1 in both vehicle and PCB-treated systems. Along with this upregulated antioxidant response, Cav-1 siRNA treated cells exhibited decreased mRNA levels of the Nrf2 inhibitory protein Keap1 in both vehicle and PCB-treated samples. Silencing Cav-1 also decreased protein levels of Nrf2 inhibitory proteins Keap1 and Fyn kinase, especially in PCB-treated cells. Further, endothelial cells from wildtype and Cav-1-/- mice were isolated and treated with PCB to better elucidate the role of functional caveolae in PCB-induced endothelial inflammation. Cav-1-/- endothelial cells were protected from PCB-induced cellular dysfunction as evidenced by decreased vascular cell adhesion molecule (VCAM-1) protein induction. Compared to wildtype cells, Cav-1-/- endothelial cells also allowed for a more effective antioxidant response, as observed by higher levels of the antioxidant genes. These data demonstrate novel cross-talk mechanisms between Cav-1 and Nrf2 and implicate the reduction of Cav-1 as a protective mechanism for PCB-induced cellular dysfunction and inflammation.

2.2 Introduction

Exposure to persistent environmental pollutants such as polychlorinated biphenyls has been linked to the induction and/or exacerbation of multiple human pathologies including diabetes and atherosclerosis[24a, 24b, 25b, 49, 148]. Specifically, coplanar polychlorinated biphenyls (PCBs) have been shown to initiate the earliest stages of atherosclerotic plaque formation, e.g., endothelial cell dysfunction and inflammation[12b, 23b, 97].

Once utilized extensively for their productive thermal and electrical capabilities, the production of PCBs and PCB mixtures, such as aroclors, was banned in the 1970s due to further data highlighting their substantive public health concerns[149]. However, PCBs continue to impact ecosystems and human health due to their environmental prevalence and persistence (chemical stability)[13b]. Additionally, the lipophilic nature of coplanar PCBs allows for their interaction with lipid membranes and lipid storage depots, leading to bioaccumulation and biomagnification through the food chain. Even today, PCB-containing products continue to be used in developing nations, further exposing humans to high occupational levels of these harmful pollutants[25a]. In countries such as the United States, the primary sources of exposure stem from air pollution and contaminated food[149a]. Although multiple categories of PCBs have been developed, dioxin-like, non-ortho-substituted coplanar PCBs such as PCB 77 and PCB 126 exhibit the highest levels of in vitro and in vivo toxicity and pro-inflammatory properties, especially in the vasculature[18].

Once believed to be an innate barrier, endothelial cells now appear to play an extremely important role in the initiation and progression of atherosclerosis[3, 21, 150]. Coplanar PCBs can further promote endothelial cell inflammation and dysfunction through caveolae lipid micro-domains[151]. Endothelial cell activation can lead to an upregulation of adhesion molecules such as Vascular Cell Adhesion Molecule-1 (VCAM-1) which promotes pro-inflammatory leukocyte infiltration and chemokine production that left unchecked can lead to the formation of foam cells and subsequent arterial blockage[152]. Coplanar PCBs can induce oxidative stress in endothelial cells and in turn cause the upregulation of pro-inflammatory proteins through an NFκB-mediated signaling cascade[12b]. Interestingly, it has been shown that PCBs may also be pro-

atherogenic by activating other pro-inflammatory pathways such as the lipid signaling domain caveolae[97].

Lipid raft microdomains known as caveolae are flask-shaped invaginations found at the lipid membrane and have been shown to play important roles in endocytosis, atherosclerosis, and environmental pollutant toxicity[23b, 93]. Caveolin-1 (Cav-1), the major structural and signaling protein involved in the caveolae pathway, has been shown through its "Cav-1 binding domain" (CBD) to interact and bind multiple other proteins, many of which are involved in inflammation and atherosclerosis[90, 92, 94]. Coplanar PCBs preferentially sequester in caveolae cellular fractions, and exposure to coplanar PCBs upregulates Cav-1 protein expression and caveolae formation[12b]. Silencing Cav-1 via siRNA technology prevents PCB-induced cytochrome P450-mediated superoxide production and subsequent endothelial activation and dysfunction[12b]. Importantly, it has been shown that aortic endothelial cells isolated from mice that lack the Cav-1 gene are protected from toxicant-induced cellular dysfunction, but the mechanism of this protection has yet to be elucidated[12a].

Physiological systems have evolved multiple signaling pathways to limit the toxicity of xenobiotics such as PCBs. The most significant regulator of redox status and homeostasis, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant pathway, has been shown to be critical in protecting endothelial cells from PCB toxicity[73b]. Nrf2 is primarily regulated by its major inhibitory protein, iNrf2 or Keap1, which promotes Nrf2's ubiquitination and maintains basal levels of Nrf2 activation. Nrf2 also can be inhibited by Fyn kinase, which can promote Nrf2 nuclear exit and degradation[153]. Nrf2 also can be directly activated via phosphorylation by kinases such as Akt and PKC delta[99a, 154]. Nrf2 can become activated by xenobiotic electrophiles, reactive oxygen species (ROS), and bioactive phytochemicals found in healthful nutrition[82a, 100, 123b]. Multiple electrophilic bioactive nutrients including components of ginseng, green tea and vegetables such as broccoli have been shown to activate Nrf2, but interestingly, different nutrients may induce Nrf2 through differing mechanisms (e.g., disruption of Keap1/Nrf2 interaction or increased phosphorylation via relevant kinases[137a, 155]. Nrf2 has been shown to be regulated through the cross-talk of multiple signaling cascade pathways such as the aryl hydrocarbon receptor (AhR) and NFκB pathways[86b, 138-139]. Many xenobiotics such as dioxins and coplanar PCBs can activate both AhR and Nrf2 simultaneously, and in fact, this concordant upregulation can be evolutionarily explained since the gene promoter for

AhR contains multiple Nrf2 binding elements (AREs) and the promoter for Nrf2 contains AhR binding sites (xenobiotic response elements)[138]. Evidence for direct cross-talk between Nrf2 and NFκB is not as well understood, but multiple studies have shown that activation of Nrf2 leads to a diminished pro-inflammatory NFκB response[156]. Interestingly, we have previously shown that the AhR is a binding partner of Cav-1[12b] which has led us to hypothesize that novel cross-talk between Cav-1 and Nrf2 could exist and that this cross-talk may help to explain mechanistically the protection from coplanar PCBs observed in Cav-1 -/- animals.

Thus, the current study has been designed to investigate mechanistically how the cross-talk between Cav-1 and Nrf2 can modulate PCB-induced cellular dysfunction. Our data provide strong evidence that there are multiple levels of Cav-1/Nrf2 cross-talk and that Cav-1 inhibits the Nrf2 antioxidant response. Thus, reduction or downregulation of endothelial Cav-1 may lead to an upregulated antioxidant response regulated by Nrf2, which could better prime a physiological system prior to toxicological insult.

2.3 Materials and Methods:

2.3.1 Materials and Chemicals

3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) was obtained from AccuStandard Inc. (New Haven, CT). VCAM-1 and Keap1 primary antibodies and horseradish peroxidase-conjugated goat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin and GST primary antibodies were purchased from Sigma (St. Louis, MO). The GST antibody used recognized native as well as denatured-reduced forms of GST protein. NQO1 primary antibody was purchased from Abcam (Cambridge, MA). Fyn kinase, P-Akt, Akt, P-PKC delta and Nrf2 primary antibodies and horseradish peroxidase-conjugated rabbit secondary antibody were purchased from Cell Signaling Technologies (Danvers, MA).

2.3.2 Cell culture and experimental media

Primary vascular endothelial cells were isolated from porcine pulmonary arteries[12a]. Cells were cultured in M199 (Gibco, Grand Island, NY), supplemented with fetal bovine serum (FBS; Gibco). EAhy.926 human endothelial cells were cultured as described previously[12c]. Endothelial cells were grown to confluence, followed by incubation overnight in medium containing 1% FBS prior to cell treatment. Stock solutions of PCB 126 were prepared in DMSO; control cultures were treated with DMSO vehicle. The levels of DMSO in experimental media were 0.05%. Porcine and human endothelial cells were treated with PCB 126 at 0.25 μM for 16 h and mouse pulmonary endothelial cells were treated with 2.5 μM for 24 h, which are established concentrations used previously in our laboratory[12a, 73b]. Porcine primary endothelial cells, Eahy.926 human endothelial cells, and isolated mouse endothelial cells contained Cav-1 protein levels (wildtype) and exhibited VCAM-1 induction due to PCB 126 treatment, as previously reported[12b, 12c]. Endothelial cells isolated from wildtype and Cav-1 -/- mice were identified by morphology, platelet endothelial cell adhesion molecule-1 (PECAM1) bead pull-down, and Von Willebrand Factor (VWF) protein expression.

2.3.3 Mouse endothelial cell isolation

Endothelial cells were isolated from Cav-1 deficient (Cav-1−/−) and wildtype mice (both genotypes were purchased from Jackson Laboratory, Bar Harbor, ME). All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facilities at the University of Kentucky. Age matched C57BL/6 mice

were used as controls because Cav-1-deficient mice are backcrossed onto C57BL/6 mice. Endothelial cells were isolated and cultured as described previously[157]. Briefly, whole lungs were homogenized in culture media containing type II collagenase and dispase. Cells were added to gelatin-coated tissue culture plates in DMEM media containing 20% FBS, heparin, antibiotics, and endothelial cell growth supplement. Endothelial cells were preferentially selected by using an antibody-coated magnetic bead mix (Invitrogen, Carlsbad, CA. Briefly, sheep anti-rat IgG beads were prepared and mixed with rat anti-mouse CD31 PECAM antibodies overnight (BD Biosciences, San Jose, CA). Bead/antibody conjugates were collected via magnetic separation and subsequently were incubated for 1 h with cells isolated from lungs. Cells were then trypsinized, transferred to a magnetic separator, and magnetically bound cells were seeded in 35mm plates. Endothelial cell isolation was confirmed by cobblestone morphology and the presence of VWF endothelial cell marker.

2.3.4 Real-time PCR

The levels of mRNA expression were assessed by real-time PCR using a 7300 Real-Time PCR System (Applied Biosystems,Foster City, CA) and SYBR Green master mix (Applied Biosystems) as described earlier[12a]. Sequences were designed using the Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences for porcine VCAM-1 and β-actin were described in earlier articles published from our laboratory[23b, 158]. Porcine NQO1 sequences were: sense, 5′-CCCGGGAACTTTCAGTATCCT-3′; and antisense, 5′-CTGCGGCTTCCACCTTCTT-3′; porcine GST-omega 1 sequences were: sense, 5′-GCTGAGCCAAGTGGGAGACA-3′; and antisense, 5′CCTCGGCCATTGAAATAGTGA-3′; Porcine Keap1 sequences were: sense, 5’ AGCTGGGATGCCTCAGTGTT-3’; and antisense, 5’-AGGCAAGTTCTCCCAGACATTC-3’; porcine GPx1 sequences were: sense, 5′-TGCCTTCAATCCGGAGGTAA-3′; and antisense, 5′GAATGGGATGACCTGGAAGTTAGT-3′; porcine MRP1 sequences were: sense, 5′-GTCCTGTTTGCTGCCTGTT-3′; and antisense, 5′AGTATGCGGTGATCTGCAGTGA-3′;

2.3.5 Cav-1 siRNA and transfection studies

Double stranded small interfering RNA targeting Cav-1 was synthesized as described previously[12c, 159]. Porcine endothelial cells and human endothelial cells were transfected with control or Cav-1 siRNA at a final concentration of 80 nM using GeneSilencer transfection reagent (Genlantis, SanDiego, CA) in OptiMEM serum free media. Cells

were incubated with the transfection mixture for 4 h, followed by the addition of 10% FBS to the cell media. Cells were used for treatments after 48 h incubation.

2.3.6 Immunoblotting

Western blot analyses for VCAM-1, β-actin, NQO1, Keap1, Fyn kinase, GST, Nrf2, P-PKC delta and P-Akt were performed as described previously[12a]. Briefly, cells were lysed in RIPA buffer, centrifuged and the protein concentrations were determined via Bradford Assay. Proteins were separated via 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 5% non-fat milk or BSA. Primary antibodies were added at a concentration of 1:1000 overnight and appropriate secondary antibodies subsequently were added for 2 h at a concentration of 1:4000.

2.3.7 Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts of endothelial cells were prepared using NE-PER nuclear extraction reagents (Thermo, Rockford, IL) according to the manufacturer's protocol. Nuclear extract concentrations were determined using Bradford reagent (Bio-Rad, Richmond, CA). DNA binding activities of Nrf2 were determined using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer's protocol. DNA-binding reactions were performed with a final volume of 20 µL buffer containing 5 µg of nuclear extract, 50 ng/µL Poly (dI•dC), and biotin end-labeled oligonucleotides. Synthetic 5'-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotides containing the antioxidant response element (ARE) sequence from the porcine NQO1 promoter (5'-TAGTCACAGTGACTCAGCGAGATTC-3') were used as described previously[159b].

2.3.8 Statistical analysis

Data were analyzed using SigmaStat software (Systat Software, Point Richmond, CA). Comparisons between treatments were made by two-way ANOVA with post-hoc comparisons of the means. To elucidate trends within groups, multiple comparison procedures were completed for tests that displayed overall significance ($p \leq 0.05$-0.1). Groups were considered significantly different with a determined p value of $p<0.05$.

2.4. Results

2.4.1 Cav-1 silencing prevents PCB-induced endothelial cell dysfunction by increasing antioxidant gene expression

Previously, we have shown that coplanar PCBs can initiate cellular dysfunction, as evidenced by an increase in VCAM-1 protein levels, in endothelial cells that contain functional caveolae[12a, 12b]. Here, we treated primary porcine vascular endothelial cells with a single low dose (0.25 μM) of PCB 126 for 16 h and saw a statistically significant upregulation of VCAM-1 mRNA expression in control siRNA treated cells (Fig. 2.1). However, this upregulation was not observed in cells treated with Cav-1 siRNA. We have previously shown that the antioxidant controller Nrf2 is critical for cellular defense against PCB toxicity, thus we hypothesized that Cav-1 silenced cells were protected through a Cav-1/Nrf2 cross-talk mechanism. Cav-1 siRNA treated cells displayed increased Nrf2- antioxidant response element (ARE) transcriptional binding (EMSA) which was most evident when comparing between baseline levels of vehicle DMSO treated groups (Fig. 2.2B). We then investigated, via real time PCR (RT-PCR), mRNA expression levels of two major Nrf2 target genes GST and NQO1 and saw statistically significant increases of expression for both genes in cells treated with Cav-1 siRNA compared to control scrambled siRNA (Fig. 2.2A). As with the EMSA results, the most drastic and significant differences between Cav-1 and control siRNA treated cells were seen in DMSO vehicle groups. Basal levels of GST expression were approximately 10 fold higher in Cav-1 siRNA treated cells but only approximately 5 fold higher in Cav-1 siRNA treated cells after exposure to PCB. NQO1 levels were also significantly higher in cells silenced for Cav-1 but to much less of a degree (Fig. 2.2A). Additionally, NQO1 basal protein levels were significantly increased in Cav-1 transfected cells after 48 h compared to control transfected cells (Supplemental. Fig. 2.1A). Finally, basal mRNA levels of two additional Nrf2 targets, glutathione peroxidase-1 (GPX1) and multi-drug resistance protein-1 (MRP1) were compared between Cav-1 and control transfected cells, and it was determined via RT-PCR that even after just 24 h of Cav-1 transfection, expression levels of both of these cytoprotective genes were doubled in cells silenced for Cav-1 (Supplemental Fig. 2.1B).

2.4.2 Decreased Cav-1 expression promotes Nrf2 activity by decreasing levels of multiple Nrf2 inhibitory proteins

Nrf2 is inhibited by multiple factors including Keap1 and Fyn kinase; therefore we investigated levels of these proteins in an attempt to elucidate further novel mechanisms of Cav-1/Nrf2 cross-talk (Fig. 2.3). To explain the upregulated Nrf2 activity in Cav-1 depleted cells, we hypothesized that levels of these inhibitory proteins necessarily would be diminished early on during PCB exposure. Thus, we treated human endothelial cells (HUVEC fusion) with control or Cav-1 targeted siRNAs and subsequently exposed the HUVEC-based cell line to 0.25 μM PCB 126 for a minimal time duration (4 h). As expected, PCB exposure caused a decrease in protein expression of the Nrf2 inhibitory proteins Keap1 and Fyn kinase in both Cav-1 and control siRNA treated cells. More importantly, at basal DMSO conditions, cells silenced for Cav-1 also displayed significantly lower levels of Keap1 and Fyn compared to DMSO-treated control siRNA cells. Exposing PCB to these Cav-1 silenced cells resulted in a statistically significant decrease in Fyn and Keap1 compared to Cav-1 silenced cells treated with DMSO. Although antibodies for Fyn and Keap1 used by our laboratory did not work considerably well in our mouse and porcine models, we did examine, via RT-PCR, Keap1 mRNA levels in Cav-1 silenced porcine cells and observed similarly significant trends as with human endothelial cell protein data (Fig. 2.8). Finally, whole livers were harvested from wildtype and Cav-1 -/- mice, and via RT-PCR it was determined that Cav-1 -/- mice expressed approximately 50% mRNA expression levels of Keap1 in the liver compared to age matched wildtype controls (n=4 for each genotype, Fig. 2.9). This observed decrease in Keap1 in whole livers of Cav-1 -/- was similar to the diminished levels observed in endothelial cells from porcine or human silenced for Cav- 1(compare to Fig. 2.3 or Fig. 2.8); which is interesting due to the fact that multiple cell types can be found in whole liver lysates).

2.4.3 Decreased Cav-1 expression promotes the phosphorylation of Akt but not phosphorylation of PKC delta.

Alternative mechanisms of Nrf2 activation have been investigated including direct phosphorylation of Nrf2 by PKC delta and Akt. To investigate the ability of Cav-1 silencing to alter either of these Nrf2 effectors, human endothelial cells were transfected with either scrambled or Cav-1 targeting siRNAs and subsequently treated with 0.25 μM PCB 126 for 4 h. Interestingly, Cav-1 silencing decreased levels of phosphorylated PKC

delta in both DMSO and PCB treatment groups, although significantly increased levels of phosphorylated Akt were seen in baseline vehicle treatment groups but not when treated with PCB (Fig. 2.4).

2.4.4 *Cells isolated from Cav-1 -/- mice are protected from PCB-induced inflammation via upregulated Nrf2 activity*

Lung endothelial cells were next isolated from Cav-1 deficient and wildtype mice of the same background and subsequently exposed to coplanar PCB 126 to confirm the importance of Cav-1/Nrf2 cross-talk in PCB-induced toxicity. VCAM-1 protein levels were measured via Western blot and quantified via densitometry. As mirrored in the siRNA experiments utilizing porcine cells, PCB 126 only increased cellular dysfunction (VCAM-1 induction) in cells isolated from C57BL/6 mice and not Cav-1 -/- mice (Fig. 2.5). We attribute this protection in Cav-1 -/- mice to the considerably higher protein levels of antioxidant enzymes such as NQO1 and GST. Cells isolated from Cav-1 -/- mice displayed approximately 10 fold and 3 fold increases in NQO1 and GST, respectively, in vehicle treated groups when compared to cells isolated from C57Bl/6 control mice (Fig. 2.5). Treating mouse endothelial cells with PCB resulted in the same trends observed in the previous porcine siRNA experiments (e.g. decreased levels of GST and higher levels of NQO1 compared to DMSO vehicle). Importantly, although an overall interactive effect was not seen, we did see a trend toward significance (P=0.072) for increased Nrf2 protein levels in cells isolated from Cav-1 -/- mice when comparing baseline DMSO groups between mouse genotypes (Fig. 2.5). Combining this finding with the EMSA results displayed in Fig. 2.2B, together, help to show that a decrease in cellular Cav-1 protein promotes upregulated Nrf2 activity that is evident in multiple species.

2.5. Discussion

There is a growing body of evidence that implicates human exposure to persistent organic pollutants (POPs) such as polychlorinated biphenyls with increased risk of inflammatory related diseases such as atherosclerosis. The endothelium is a major target of PCB toxicity due to, e.g., constant low dose release from adipose fat stores caused by dynamic equilibrium and/or weight loss. Understanding the multiple signaling pathways involved and their cross-talk is a critical step in designing

preventative and therapeutic methods to counteract pollutant-induced endothelial cell dysfunction and inflammation.

The importance of caveolae, lipid rafts, and Cav-1 in inflammation and heart disease has become a source of much research, but until recently, cross-talk between caveolae-related proteins and other factors has not been elucidated. Now, evidence points to interactions between Cav-1, AhR, and Nrf2, all proteins involved in inflammation and toxicant-induced disease[12b, 140, 160]. This work shows that a low dose of coplanar PCB 126 is sufficient to induce a pro-inflammatory endothelial cell environment and that loss of Cav-1 is protective and results in increased Nrf2 activity via multiple novel cross-talk mechanisms.

Coplanar PCBs have been shown to induce early stages of atherosclerosis via altering the cellular redox balance. Treatment with coplanar PCBs such as PCB 77 or PCB 126 has been shown in endothelial cells to increase oxidative stress, and inflammatory markers such as MCP-1 expression, and monocyte adhesion, but protection is seen in cells and mice deficient for the Cav-1 gene[12a, 12b, 23b]. The pro-inflammatory effects of PCBs may be linked to their uptake via caveolae, and the work described here suggests that Cav-1 deficiency protects against PCB-induced cellular dysfunction by allowing for a more effective Nrf2-controlled antioxidant response. Previously it was believed that lack of Cav-1 protected these cells and animals from PCB toxicity through an impaired PCB uptake mechanism. Previous work in our laboratory further has shown that PCBs preferentially sequester in the caveolae fraction of endothelial cells and PCB treatment increases the formation of functional caveolae at the lipid membrane[12b]. Disruption of functional caveolae and/or downregulation of Cav-1 may protect against toxicant-induced cytotoxicity via multiple mechanisms including altering the uptake of contaminants, altering toxicity kinetics and, as described in this work, inducing an upregulated Nrf2 antioxidant response. However, specific uptake mechanisms of PCBs may vary in different cell types and among congener of PCBs which may implicate the importance of the upregulated Nrf2 response over other mechanisms of protection in certain cell types and cellular conditions[161]. Loss of Cav-1 has been shown by our laboratory and others to cause an upregulated Nrf2 antioxidant response[159b], but until recently, mechanisms of this cross-talk had not been elucidated. Two groups have now shown that Nrf2 can be added to a growing list of proteins that can be inhibited by Cav-1 via direct binding within the cell[140, 160]. These studies,

however, did not highlight other possible mechanisms of cross-talk such as decreased Keap1 or Fyn kinase levels in Cav-1 diminished cells. Here, we show that protein levels of Keap1 and Fyn are diminished in cells silenced for Cav-1, which may help to mechanistically explain the observed upregulated Nrf2 response. Keap1 has been indicated as the major inhibitor of Nrf2 by promoting ubiquitination and subsequent proteosomal degradation. Very little is known however about cellular regulation of Keap1 expression levels, especially during transcription. Studies have shown that Keap1 levels can be decreased by epigenetic mechanisms as well as endogenous microRNAs, but future work is required to clarify the mechanism connecting diminished Cav-1 levels with decreased Keap1 expression[162]. It is important to mention that some groups have shown that loss of Cav-1 in certain cell types, especially in a cancer environment, promotes oxidative stress and can drastically alter tumor recurrence[163]. In our endothelial cell models, though, we have not observed an increase in ROS levels with Cav-1 siRNA treatment and have only observed PCB-induced cellular dysfunction in mice and endothelial cells that have wildtype levels of Cav-1[12b]. It will be important to create endothelial specific Cav-1 -/- mice in the future to better understand important connections between endothelial Cav-1, oxidative stress levels, Nrf2, and protection from xenobiotic-induced toxicity.

Recently, alternative mechanisms of Nrf2 activation have been described including direct phosphorylation by PKC delta and Akt. Articles have implicated Cav-1 as a direct inhibitor of multiple PKCs, and loss of Cav-1 has resulted in increased PKC auto-phosphorylation and activation[164]. Interestingly, we did not observe this trend with the PKC isotype that has been shown to directly activate Nrf2 (Fig. 2.4). Some groups have shown, however, that activation of PKC isoform delta can be detrimental to the vasculature and can play an important role in the promotion of cardiovascular diseases, indicating that an increase in activated PKC delta may not be all positive[165]. Activated Akt, however, was significantly increased in cells treated with Cav-1 siRNA. An interaction between Cav-1 and Akt has been implicated previously, but more work is necessary to determine if this observed increase in Akt can be correlated with Nrf2 activation[166]. Future experiments should also investigate Cav-1's role in other cellular Nrf2-related kinases such as phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), ER-localized pancreatic endoplasmic reticulum kinase (PERK), and protein kinase CK2[167]. Here, we add to a growing body of evidence that cross-talk

between Cav-1 and Nrf2 exists and that multiple mechanisms of cross-talk may be evident (Fig. 2.6).

Downregulation of endothelial Cav-1 may prove to be an effective modulator of toxicant-induced disease as well as other pathologies hallmarked by chronic inflammation. We have shown previously that healthful nutrition, which is high in bioactive food components such as polyphenols and omega-3 polyunsaturated fatty acids, can protect against PCB-induced cellular dysfunction[73b, 116a, 155a]. Laboratories have shown that these nutrients may protect through multiple mechanisms including disruption of functional caveolae, increased Nrf2 activity, and decreased NFκB activation[73b, 115b, 116b, 126, 158]. It is plausible to hypothesize that the cross-talk between Cav-1 and Nrf2 pathways is critical for nutritional and/or pharmacological modulation of toxicant-induced disease. For example, statin treatment (simvastatin) can downregulate Cav-1 protein levels and activate endothelial nitric oxide synthase (eNOS)[168]. Also, eNOS upregulation via statins was not as profound in Cav-1 deficient human endothelial cells[169]. To create more targeted and effective nutritional and pharmacological therapeutics, more work is needed to better elucidate the impact on the increasingly complex cross-talk between caveolae and Nrf2 (Fig. 2.6).

Our data suggest that cross-talk between Cav-1 and Nrf2 exists and may be more complex than described previously. Cav-1 inhibits Nrf2 activation, and decreasing Cav-1 levels increases the expression of Nrf2 target genes. This increased induction of protective genes may help to explain why cells and mice lacking Cav-1 are protected against pro-inflammatory polychlorinated biphenyl exposure. Our studies provide concordant findings from experiments using cellular models from three different mammalian species, i.e., pig, mouse, and human, suggesting some relevance of our findings to human health and risk assessments. Caveolae and Nrf2-related proteins may prove to be important targets for effective nutritional and/or pharmacological protection against the toxicity of pro-inflammatory xenobiotics.

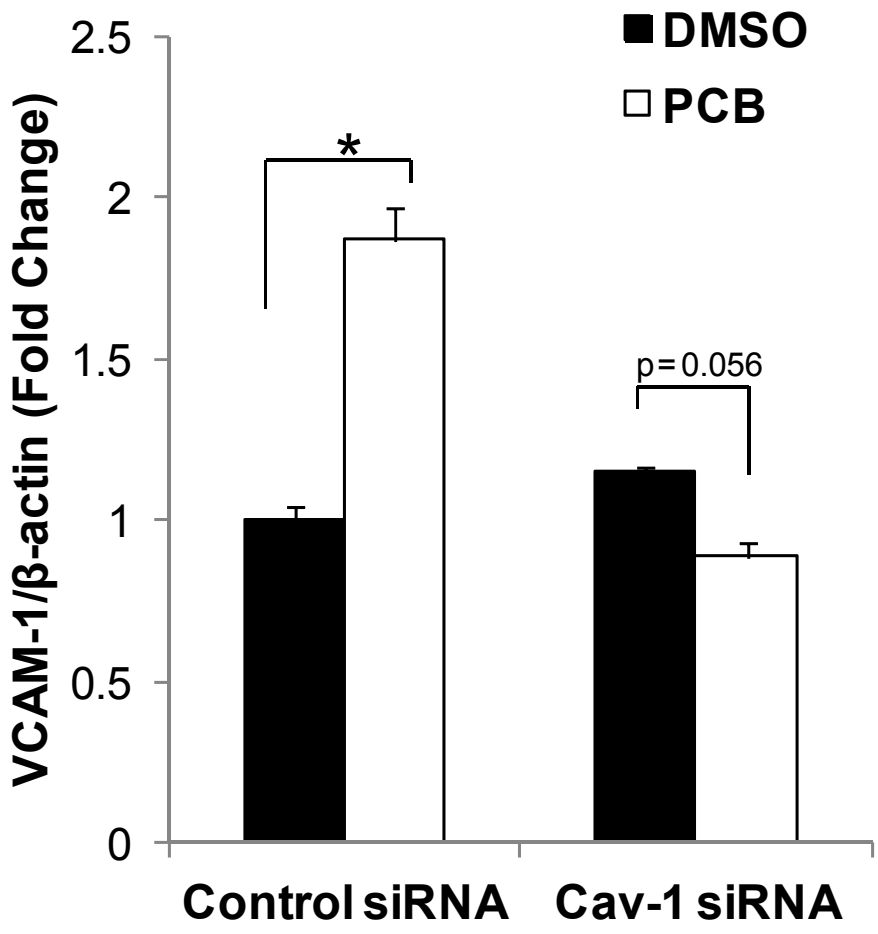

Fig. 2.1. Expression of VCAM-1 mRNA in porcine vascular endothelial cells exposed to coplanar PCB 126. Cells were transfected with either scrambled control or Cav-1 targeted siRNA for 48 h and subsequently exposed to PCB 126 at a concentration of 0.25 μM for 16 h. mRNA levels were measured using real-time PCR. Results were normalized to β-actin and are depicted as fold change compared to control siRNA DMSO treatments. Results represent the mean± SEM (n=2 for each treatment group). PCB 126 increases VCAM-1 mRNA levels only in endothelial cells with Cav-1 (*$p<0.05$).

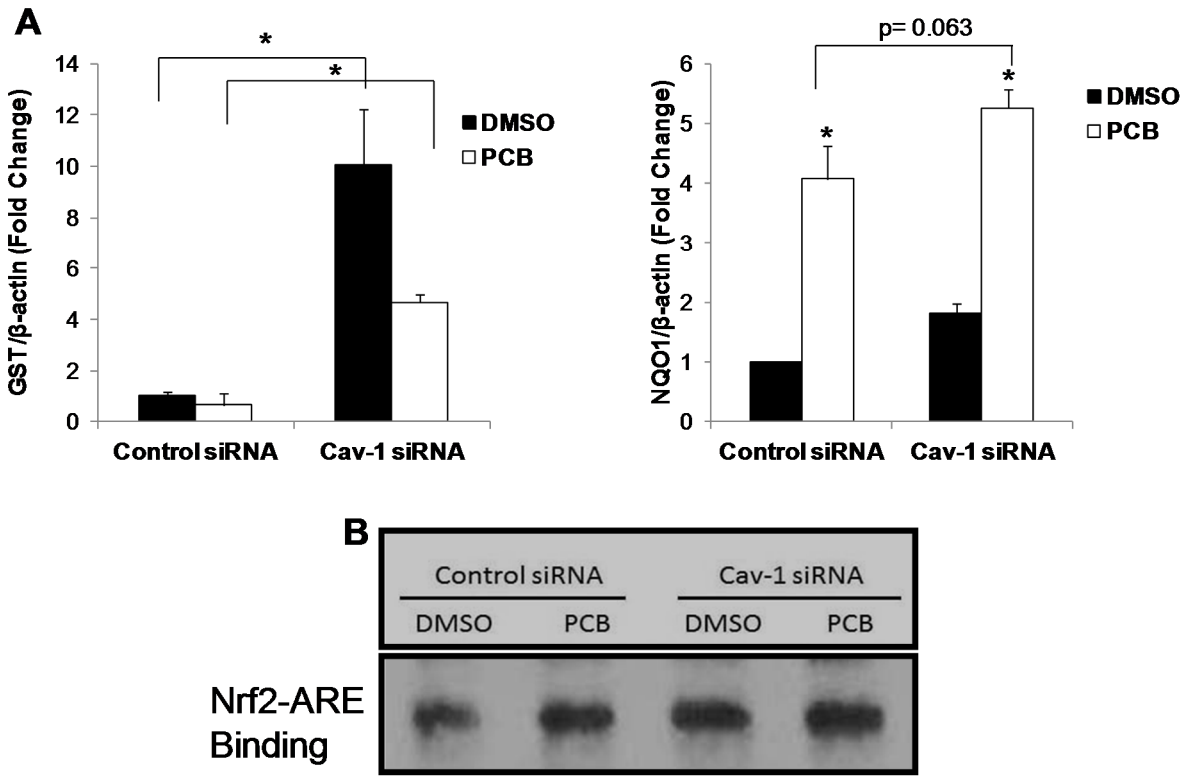

Fig. 2.2. Expression of antioxidant enzymes and Nrf2 transcriptional binding in cells silenced for Cav-1. Porcine endothelial cells were transfected with either scrambled control or Cav-1 targeted siRNA for 48 h and subsequently exposed to PCB 126 at a concentration of 0.25 μM for 16 h. mRNA levels of GST and NQO1 were measured using real-time PCR. Results were normalized to β-actin and graphed as fold change compared to control siRNA DMSO treatments. Results represent the mean± SEM (n=2 for each treatment group). (A) Cells silenced for Cav-1 display increased mRNA levels of GST in both vehicle and PCB treated groups compared to control siRNA transfected cells (*$p<0.05$). GST levels were diminished in Cav-1 siRNA transfected cells when exposed to PCB, but these levels were still statistically significantly higher than control siRNA transfected cells exposed to PCB (*$p<0.05$). Also, PCB treatment increased the expression of NQO1 in both Cav-1 siRNA and control siRNA groups (*$p<0.05$), and there was a significant trend of increased expression in cells silenced for Cav-1 ($p=0.063$). (B) Cells silenced for Cav-1 display increased levels of Nrf2 – ARE binding as determined by EMSA compared with control siRNA transfected cells.

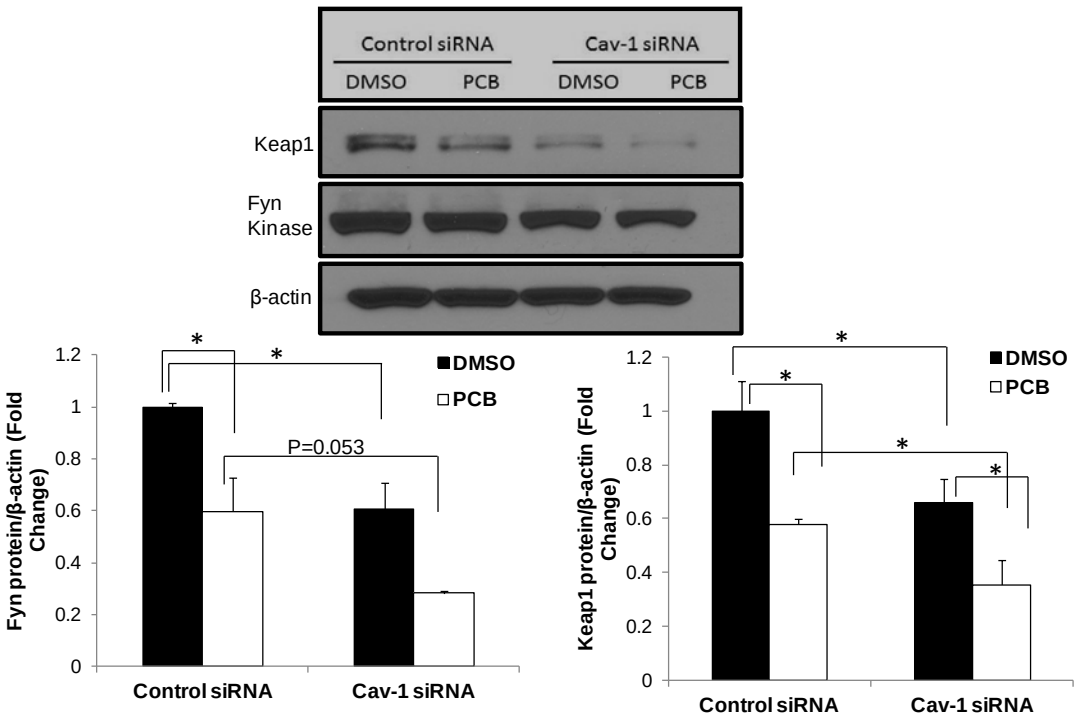

Fig. 2.3. Expression of Nrf2 inhibitory proteins is decreased in cells silenced for Cav-1. Human endothelial cells were transfected with either scrambled control or Cav-1 targeted siRNA for 48 h and subsequently exposed to PCB 126 at a concentration of 0.25 µM for 4 h. Protein levels of Keap1 and Fyn kinase were measured via western blot. Results were normalized to β-actin and graphed as fold change compared to control siRNA DMSO treatments. Results represent the mean±SEM (Fyn: n=2, Keap1: n=3 for each treatment group). Cells silenced for Cav-1 display decreased protein levels of Keap1 and Fyn in both vehicle and PCB treated groups compared to control siRNA transfected cells (*$p<0.05$)**.** PCB treatment also significantly decreased Fyn and Keap1 levels within transfection groups (*$p<0.05$)**.** Western blot above shows representative sample of visual results.

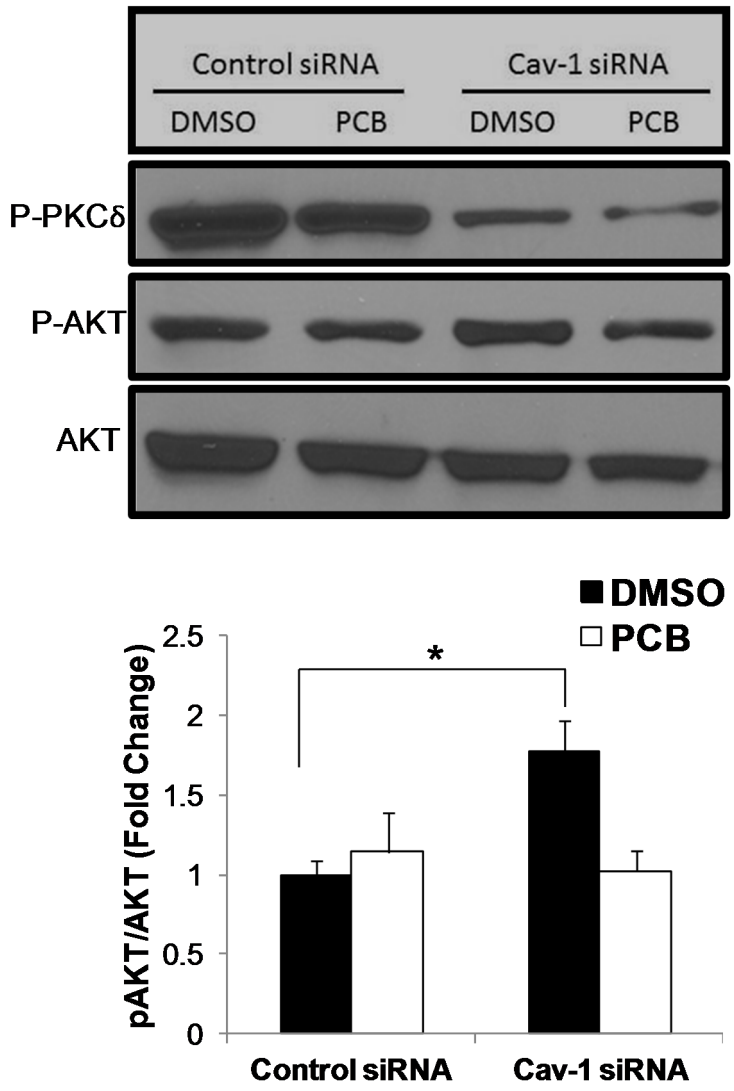

Fig. 2.4. Expression of phosphorylated Akt and PKC delta proteins in human endothelial cells exposed to coplanar PCB 126. Cells were transfected with either scrambled control or Cav-1 targeted siRNA for 48 h and subsequently exposed to PCB 126 at a concentration of 0.25 μM for 4 h. Protein levels of P-Akt and P-PKC delta were measured via Western blot. Results were normalized to β-actin and depicted as fold change compared to control siRNA DMSO treatments. Results represent the mean±SEM (n=3 for each treatment group). Cells silenced for Cav-1 display decreased protein levels of P-PKC delta in both vehicle and PCB treated groups. Cells targeted with Cav-1 siRNA and treated with DMSO vehicle had significantly increased levels of P-Akt compared to control transfected cells also treated with DMSO (*$p<0.05$). Western blot above shows representative sample of visual results.

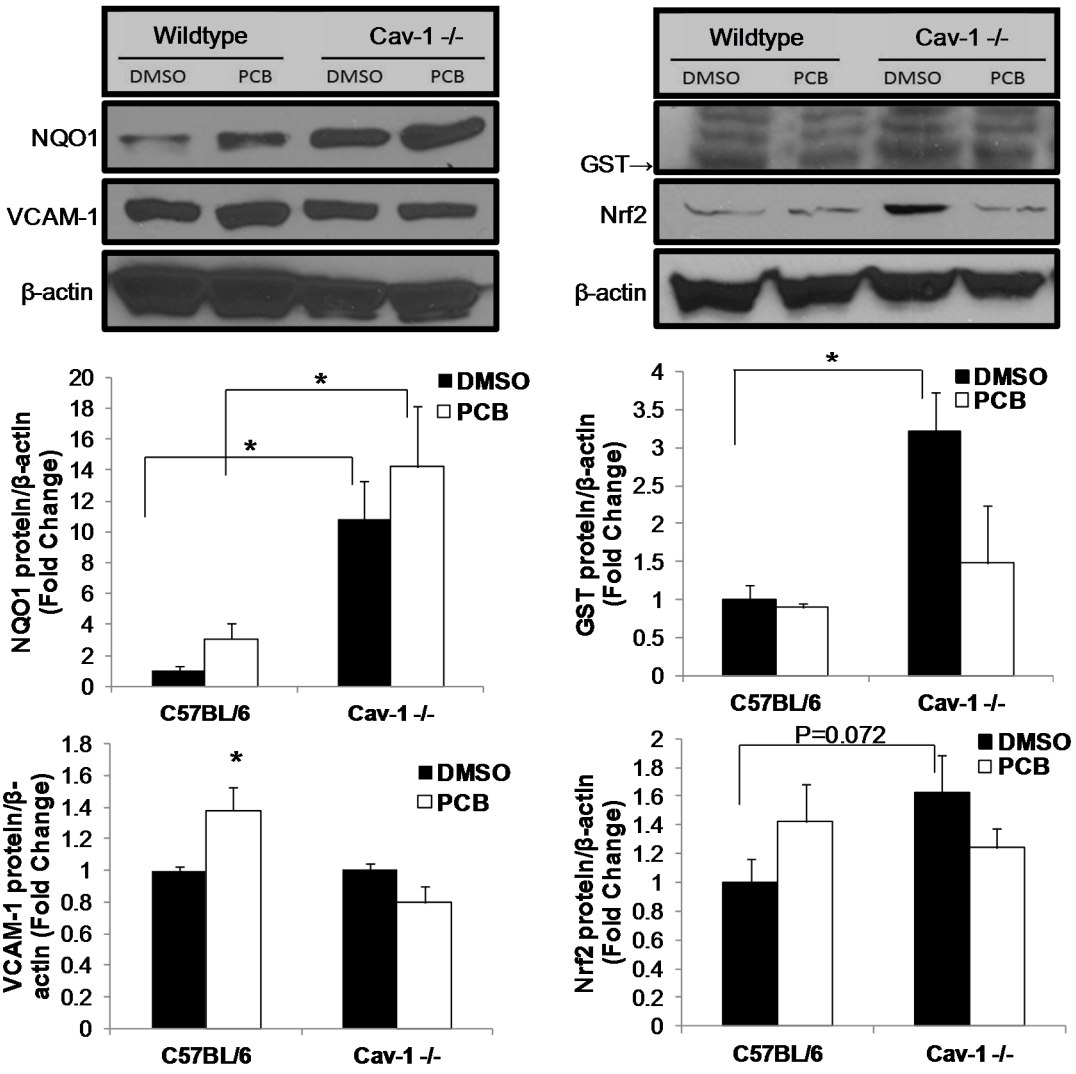

Fig. 2.5. Confirmation of Cav-1 Nrf2 cross-talk in cells isolated from Cav-1 -/- mice. Endothelial cells from Cav-1 -/- and age matched wildtype control mice were isolated and subsequently exposed to PCB 126 at a concentration of 2.5 μM for 24 h. Protein levels of NQO1, VCAM-1, GST and Nrf2 were measured via Western blot. Results were normalized to β-actin and depicted as fold change compared to control siRNA DMSO treatments. Results represent the mean±SEM (NQO1, GST, Nrf2: n=3, VCAM-1: n=2 for each treatment group). Cells isolated from Cav-1 -/- mice did not display PCB-induced VCAM-1 upregulation and showed significantly increased levels of NQO1 (*p<0.05). Cells isolated from Cav-1 -/- mice displayed significantly higher levels of GST under DMSO vehicle conditions compared to wildtype cells treated with DMSO (*p<0.05). Also, cells isolated from Cav-1 -/- mice displayed a trend toward being significantly increased compared to vehicle treated control cells. Western blots above show representative samples of visual results.

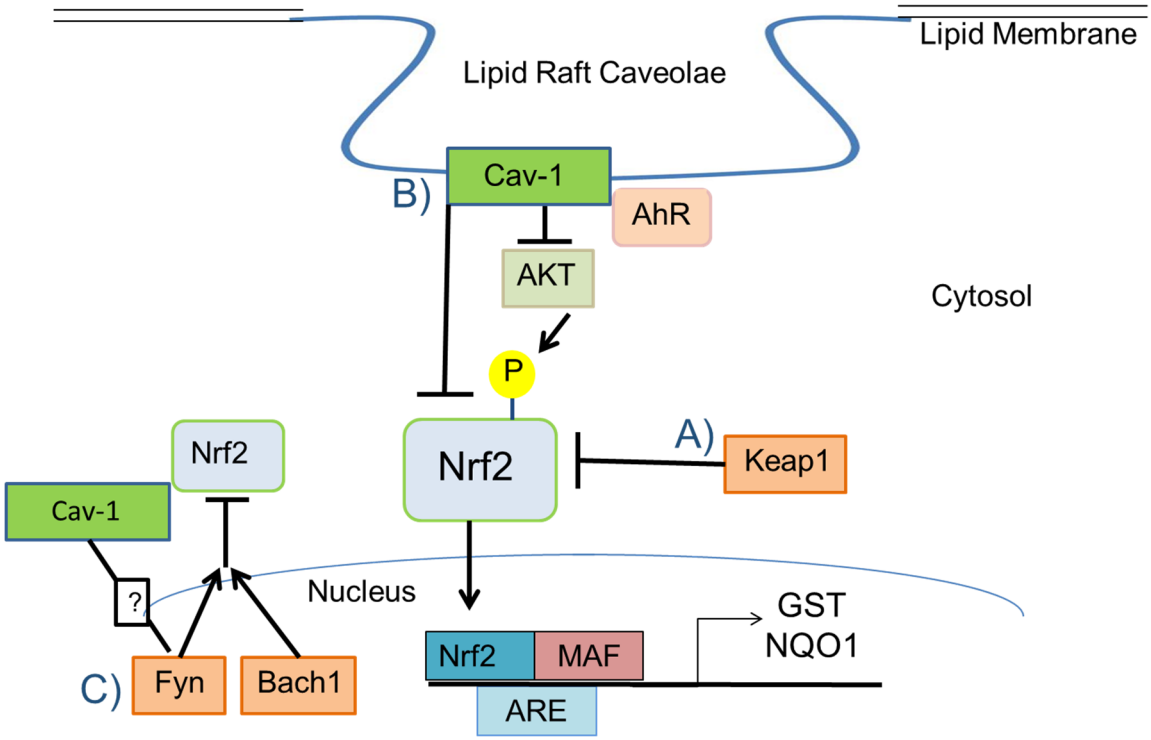

Fig. 2. 6. The induction of Nrf2 is controlled by multiple inhibitory proteins and a complex regulatory mechanism. A) Keap1 is the major inhibitory protein of Nrf2 and facilitates Nrf2's ubiquitination and destruction. B) Caveolin-1, the major structural protein of lipid raft caveolae, binds and inhibits Nrf2. Also, caveolae concentrate other proteins involved in Nrf2 activation such as Akt which is known to phosphorylate and activate Nrf2. Cav-1 is also a binding partner of the Aryl hydrocarbon receptor which is known to bind *cis*-acting sequences in the Nrf2 promotor. C) Upon Nrf2 activation and subsequent translocation to the nucleus, additional regulatory mechanisms exist to remove Nrf2. Direct phosphorylation from Fyn kinase or Bach1 decreases the Nrf2 nuclear pool. Cav-1 may also regulate Fyn and/or Bach1 levels via an unknown mechanism. If Nrf2 evades cellular destruction and remains in the nucleus, the transcription factor can bind co-activators, bind *cis*-acting antioxidant response elements on DNA, and upregulate a battery of antioxidant related proteins such as GST and NQO1.

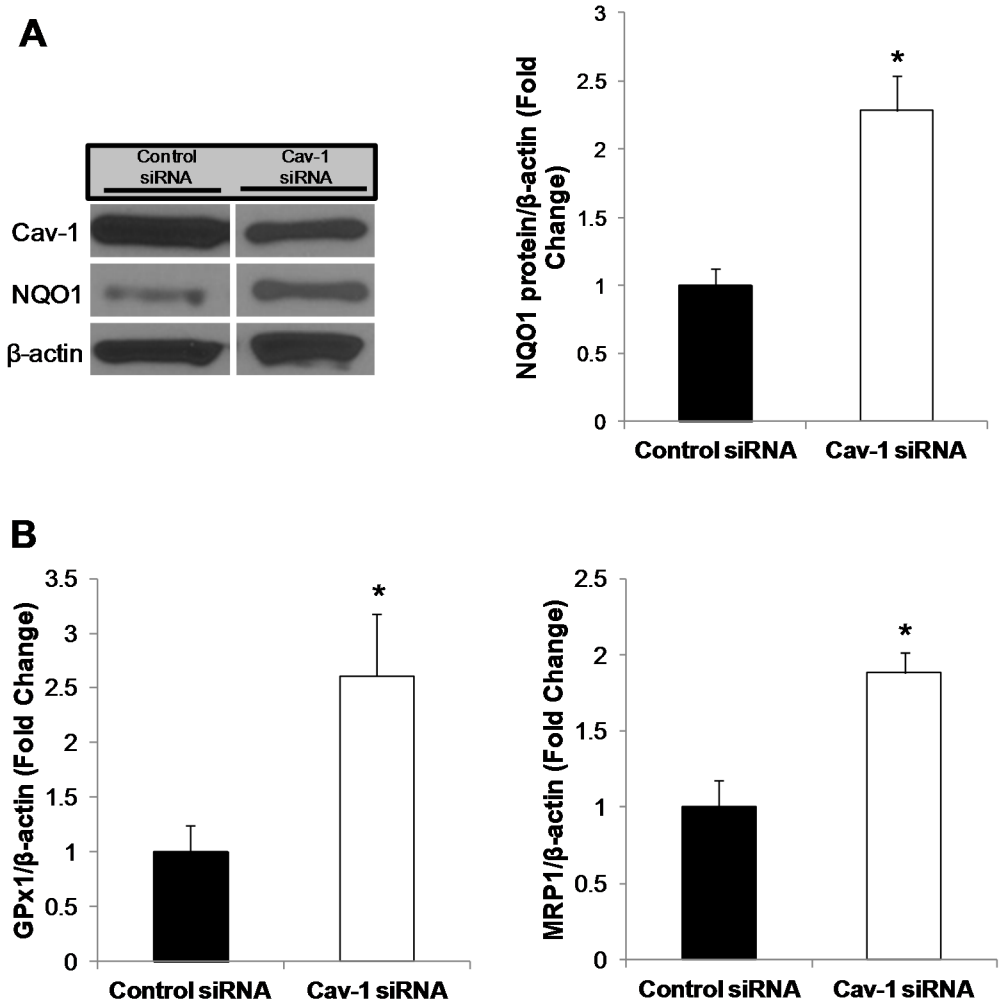

Fig. 2.7. Expression of antioxidant enzymes in cells silenced for Cav-1. Porcine endothelial cells were transfected with either scrambled control or Cav-1 targeted siRNA for (A) 48 h or (B) 24 h. (A) NQO1 protein levels were measured via Western blot. Results were normalized to β-actin and depicted as fold change compared to control siRNA groups. Results represent the mean±SEM (n=5-6 for each group). Silencing of Cav-1 for 48 h increases basal levels of NQO1 protein (*$p<0.05$). Western blots above show representative samples of visual results. (B) mRNA levels of the Nrf2 targets Glutathione Peroxidase-1 (GPx1)and Multi-drug resistance protein 1 (MRP1) were measured using real-time PCR. Results were normalized to β-actin and graphed as fold change compared to control siRNA groups. Results represent the mean± SEM (n=7-8 for each group). Cells silenced for Cav-1 display increased mRNA levels of GPx1 and MRP1(*$p<0.05$).

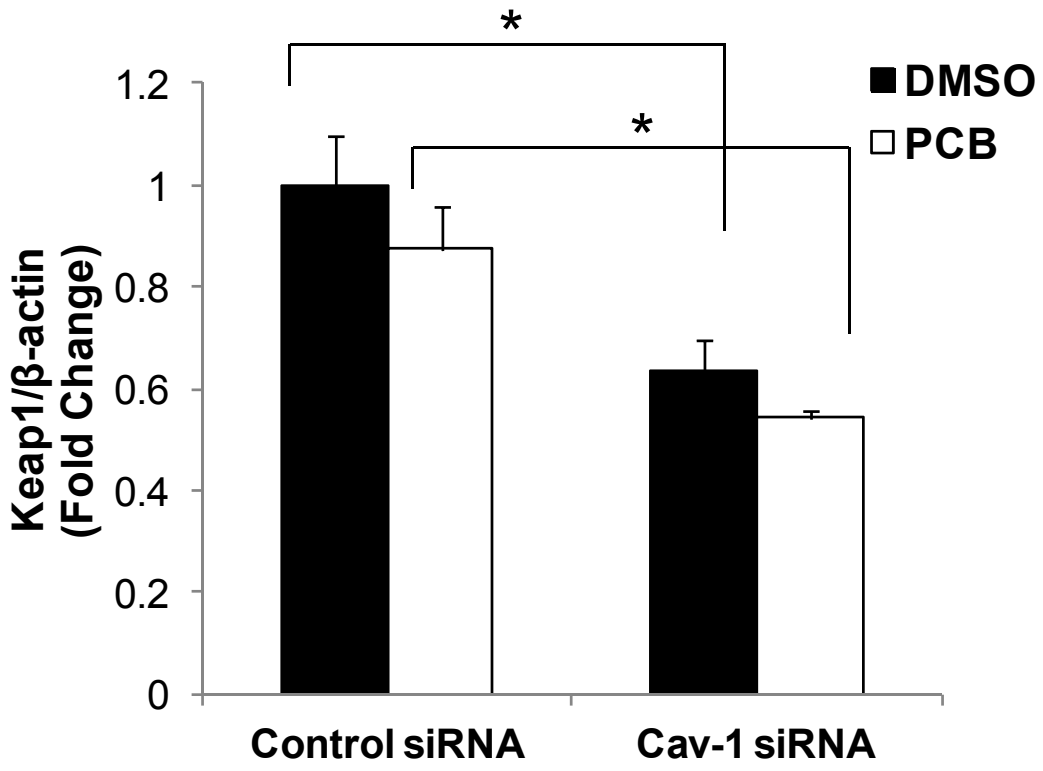

Fig. 2.8. Expression of Keap1 mRNA in porcine endothelial cells exposed to vehicle and coplanar PCB 126. Cells were transfected with either scrambled control or Cav-1 targeted siRNA for 48 h and subsequently exposed to PCB 126 at a concentration of 0.25 μM for 16 h. mRNA levels of Keap1 were measured via RT-PCR. Results were normalized to β-actin and depicted as fold change compared to control siRNA DMSO treatments. Results represent the mean±SEM (n=3 for each treatment group). Silencing Cav-1 resulted in decreased Keap1 mRNA expression levels in both vehicle and PCB treated cells (*$p<0.05$).

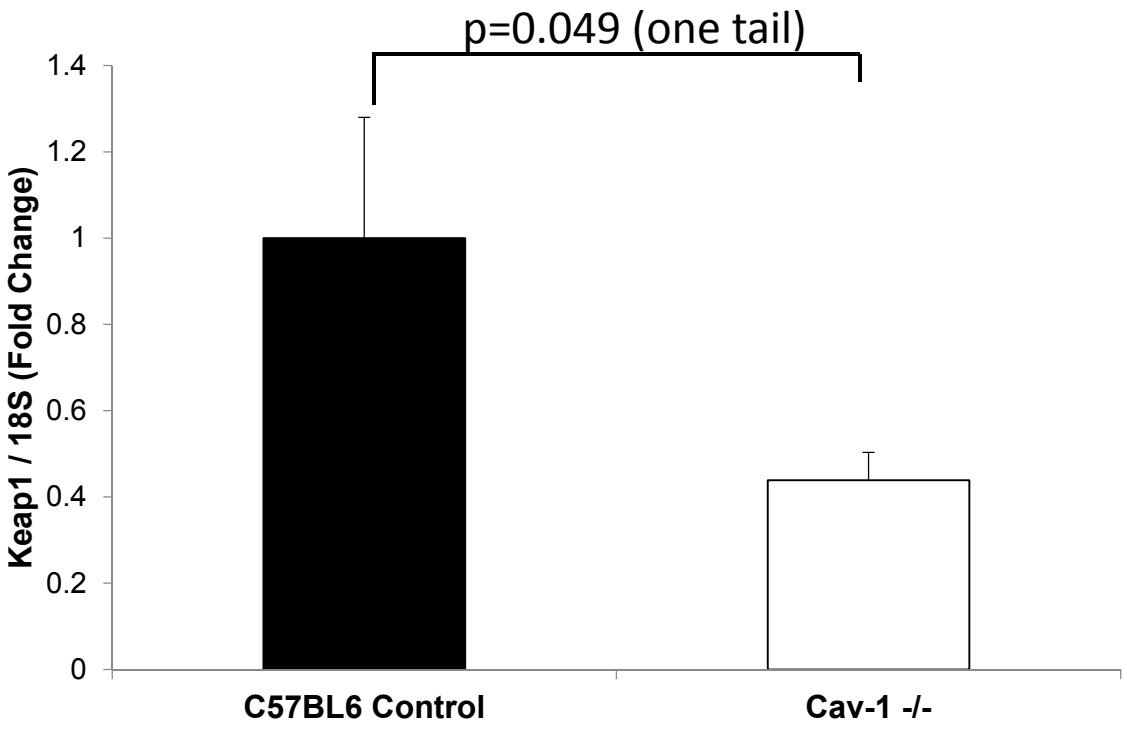

Fig. 2.9. Expression of Keap1 mRNA in livers from age matched wild-type C57BL6 mice and Cav-1 -/- mice. mRNA levels of Keap1 were measured via RT-PCR. Results were normalized to 18S and depicted as fold change compared to control mice. Results represent the mean±SEM (n=4 for each treatment group). Loss of Cav-1 resulted in decreased Keap1 mRNA expression levels (p=0.049 for one tailed t-test and p=0.098 for two tailed t-test).

Chapter 3. Green tea diet decreases PCB 126-induced oxidative stress in mice by upregulating antioxidant enzymes[129b]

3.1 Synopsis

Superfund chemicals such as polychlorinated biphenyls pose a serious human health risk due to their environmental persistence and link to multiple diseases such as atherosclerosis, diabetes, and cancer. Selective bioactive food components such as flavonoids/polyphenols have been shown to ameliorate PCB toxicity, but primarily in an *in vitro* setting. Here, we show that mice fed a green tea-enriched diet and subsequently exposed to multiple doses of coplanar PCB exhibit decreased overall oxidative stress levels primarily due to the upregulation of a battery of antioxidant enzymes. C57BL/6 mice were fed a low fat diet supplemented with green tea extract for 12 weeks and exposed to 5 μmol PCB 126/kg mouse weight (1.63 mg/kg-day) on weeks 10, 11 and 12. HPLC-MS/MS plasma analysis showed that mice supplemented with green tea extract and subsequently exposed to PCB displayed a decrease in overall F_2-Isoprostane levels compared to animals on a control diet exposed to PCB. Livers were collected and harvested for both mRNA and protein analyses, and it was determined that many genes transcriptionally controlled by the aryl hydrocarbon receptor (AhR) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) proteins were upregulated in PCB-exposed mice fed the green tea supplemented diet. An increased induction of genes such as SOD1, GSR, NQO1 and GST in these mice (green tea plus PCB) may explain the observed decrease in overall oxidative stress. A diet supplemented with green tea allows for an efficient antioxidant response in the presence of PCB 126 which supports the emerging paradigm that healthful nutrition may be able to bolster and buffer a physiological system against the toxicities of environmental pollutants.

3.2 Introduction

The contamination of soil and groundwater aquifers by toxic chlorinated organic compounds at Superfund sites, e.g., polychlorinated biphenyls (PCBs) and trichloroethylene (TCE), is a pervasive environmental problem with serious public health consequences[170]. PCBs are persistent organic pollutants found in soil, air, and water, and a major source of human exposure to PCBs is from dietary intake of contaminated foods. Because PCBs are lipid soluble, they readily accumulate in human tissues, thus increasing human health concerns[27]. For example, results from the recent Aniston Community Health Survey observed a significant correlation between PCB levels and diabetes[36f], and circulating levels of PCBs were associated with atherosclerotic plaques[171]. Prenatal exposure to PCBs also may be associated with increased weight in children[172]. The liver is one tissue particularly vulnerable to PCB-induced toxicity because it is the primary organ associated with detoxification, and there is strong evidence from NHANES data that PCBs are associated with liver disease in humans[173].

There is evidence that nutrition can modulate the toxicity of environmental pollutants[174] and thus affect the vulnerability to environmental insults and compromised health. For example, PCBs can act as diet-dependent obesogens when administered with a high-fat diet, and thus worsen nonalcoholic fatty liver disease[175]. We have previously shown that PCB insult can modify lipid metabolism while dietary fat supplementation can ameliorate these negative effects[112]. For example, we have shown that PCB exposure increases neutral lipid staining in LDL-R−/− mice fed a corn oil-enriched diet (i.e., a diet rich in omega-6 fatty acids), indicating increased inflammation, while inflammation was decreased in mice fed an olive oil-enriched diet. Additionally, omega-3 fatty acids derived from fish oil are protective and reduce PCB-induced toxicity in endothelial cells[126;116a]. Similarly, antioxidant nutrients such as dietary flavonoids can protect against endothelial cell damage mediated by these persistent organic pollutants[128a;73b]. This is important since coplanar PCBs (e.g., PCB 126) exert their toxicity primarily through activation of the aryl hydrocarbon receptor (AhR) and subsequent uncoupling of cytochrome P450 1A1 (CYP1A1), which can be a source of oxidative stress[176;177].

Mammalian cells are constantly bombarded with endogenous and exogenous sources of free radicals which tip the cellular balance towards an overall oxidative stress condition. To counteract the ubiquitous nature of reactive oxygen species (ROS), such

as superoxide ($O^{\cdot -}$), and reactive intermediates, such as hydrogen peroxide (H_2O_2), mammalian cells have evolved intricate and interrelated protein defenses that can work efficiently to limit the detrimental effects of these toxic molecules. PCBs have been shown to cause oxidative stress primarily through a CYP1A1 uncoupling mediated mechanism[178]. Production of $O^{\cdot -}$ and related ROS triggers an upregulation of a battery of antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase (GSR), glutathione transferases (GST), thioredoxins (Trx) and thioredoxin reductases (TrxR)[179]. These proteins work in concert to either catalyze the transformation of ROS to benign molecules such as water and molecular oxygen or to reactivate enzymes, usually by catalytic reductions (e.g. TrxR reduces oxidized Trx to its active form[180]). Such an interconnected system requires the crosstalk of multiple regulatory pathways including AhR and Nrf2 to function as intended (see Fig. 3.1).

The aryl hydrocarbon receptor (AhR) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factors work together to detoxify xenobiotics and to upregulate the antioxidant response. Upon activation via endogenous ligands, such as arachidonic acid metabolites, or xenobiotics, such as dioxin (TCDD) or coplanar PCB 126, the AhR translocates to the nucleus, binds consensus *cis*-acting sequences known as dioxin or xenobiotic response elements (DRE or XRE) and facilitates the upregulation of multiple genes, especially those related to phase II detoxification (e.g. CYP1A1 and UDP-glucuronosyl S-transferases)[181]. Certain environmental toxicants with relatively long half-lives, such as TCDD and PCB 126, can promote sustained AhR activation resulting in chronic low levels of oxidative stress and inflammation[182]. The Nrf2 pathway shares many target genes with AhR, but is generally regarded as more of a redox sensor, as its dissociation with inhibitory proteins (e.g. Keap1) and subsequent transactivation is promoted by ROS and electrophiles[82a]. Binding of Nrf2 to consensus antioxidant response elements (ARE) upregulates a battery of protective genes including cytochrome P450s, GSTs and NAD(P)H dehydrogenase [quinone] 1 (NQO1)[136a]. Nrf2 is a critical mediator of oxidative stress and xenobiotic toxicity as evidenced by multiple studies involving Nrf2 KO mice[183,184]. It appears that the interrelatedness of Nrf2 and AhR pathways is not a coincidental occurrence as recently intimate cross-talk between the two xenobiotic related proteins has been illustrated[138]. In fact, AhR and Nrf2 promoter gene sequences contain binding sites for one another and in instances where either is absent (e.g., KO) a non-optimal protective response occurs[139]. Importantly,

bioactive nutrients such as tea catechins may work through both Nrf2- and AhR-mediated mechanisms to prevent toxicant-induced global inflammation[73b].

We have demonstrated previously that the tea catechin epigallocatechin-3-gallate (EGCG) can protect against vascular endothelial cell activation by coplanar PCBs[73b, 158], and that EGCG can inhibit AhR-regulated genes and induce Nrf2-regulated antioxidant enzymes, thus providing protection against PCB-induced inflammatory responses in cultured endothelial cells[73b]. EGCG also can inhibit oxidative damage and attenuate carbon tetrachloride-induced hepatic fibrosis[185]. Mechanisms responsible for EGCG-induced protection against environmental pollutants are not fully understood. In the current study we provide evidence that green tea extract, composed primarily of EGCG (see Table 3.3), can decrease oxidative stress in livers of mice exposed to PCB 126 and that part of the protection is due to induction of antioxidant genes. Thus, diet supplementation with green tea may allow for an efficient antioxidant response to buffer against toxicities of environmental pollutants in humans[129a].

3.3. Materials and Methods

3.3.1 Animals, diets, and dosing treatments

Forty C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 2 months of age and evenly assigned to the following experimental groups: control diet (10% kcal as fat) + vehicle, control+1% green tea extract (GTE) + vehicle, control + PCB 126, control+1% GTE + PCB 126. The control diet was purchased from Research Diets, Inc. (New Brunswick, NJ, catalog number D12450B). Sunphenon 30S-O organic green tea extract (lot number: 105131, containing 37.4% total polyphenols, 32.0% total catechins, and 5.3% caffeine) was obtained from Taiyo International Inc. (Minneapolis, MN) and incorporated into the control diet formulation, as described in Table 3.3. Green tea extract treatment amounts per body weight coincide with approximately 4 cups of tea (~200 ml/cup) per day in humans[186]. Mice were fed the control and GTE-supplemented diets for 12 weeks and were gavaged with PCB 126 (5 μmol/kg mouse) or vehicle (stripped safflower oil; Acros Chemical Company, Pittsburgh, PA) in weeks 10, 11, and 12. The PCB 126 gavage concentration was chosen based on observations in preliminary studies where gavage of 5 μmol/kg PCB 126 showed pro-inflammatory responses in C57/BL6 mice but not wasting syndrome.

3.3.2 Blood and tissue harvesting

In this study, we examined the role that green tea extract catechins play in altering oxidative stress and inflammation following insult with environmental pollutants (i.e., PCB 126). 24 h after week 12 treatment, mice were euthanized with CO_2 and quickly exsanguinated. 10X ethylenediaminetetraacetic acid (EDTA) was added to collected blood samples, briefly mixed, and centrifuged at 5000 g for 5 min at 4 °C to separate blood plasma. Plasma samples were frozen in liquid nitrogen and stored at -80 °C until processing. Livers were harvested, weighed, divided in half, and frozen in liquid nitrogen for protein studies or stored in RNA*later* solution (Life Technologies, Grand Island, NY) at 4 °C for 24 h then -80 °C prior to mRNA analysis.

3.3.3 Plasma PCB and isoprostane analysis

PCB 126 and one of its metabolites were extracted from plasma samples to determine systemic PCB and metabolite concentrations and correlate these findings to potential PCB-induced oxidative stress as well as the role of green tea extract in mitigating these effects. PCB 126 and its hydroxy metabolites were isolated from plasma samples (plus 10 µM 13C12-labeled PCB 126 internal standard (IS), Cambridge Isotope Laboratories, Tewksbury, MA) through extraction with acetonitrile and subsequent sonication and centrifugation at 15,000 rpm for 5 min to pellet plasma debris. Supernatants were dried under N_2 and reconstituted in 99:1 methanol:dI H_2O solvent mixture with 0.5% formic acid and 0.1% 5 M ammonium formate.

Measurement of F_2-Isoprostanes (F_2-IsoPs) provides one of the most reliable assessment methods for oxidative stress *in vivo*[187]. For F_2-IsoP analysis, plasma samples were added to 5:1 ethyl acetate: methanol + 0.5% acetic acid (v/v) + 10 µM 8-iso-PGF2α-D4 (internal standard, Cayman Chemical, Ann Arbor, MI), vortexed briefly, and centrifuged to pellet plasma debris. Supernatants were transferred and dried under N_2 prior to reconstitution in methanol and addition of acetic acid for subsequent solid phase extraction (SPE).

Reconstituted F_2-IsoP samples were loaded onto pre-conditioned Supel-Select HLB SPE columns (Sigma-Aldrich, St. Louis, MO) and washed with 0.5% acetic acid followed by washing with 0.5% acetic acid containing 20% methanol. Columns were eluted with methanol, eluent was evaporated to dryness with N_2, and samples were reconstituted with 50:50 methanol:dI H_2O.

Plasma PCB 126 and a hydroxy metabolite as well as extracted plasma F_2-IsoPs were analyzed using a Shimadzu ultra fast liquid chromatography (UFLC) system coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. MRM transitions monitored: 325.9/256.1, 325.9/254.1, and 325.9/184 for PCB 126; 338/268.1, 338/196.1, and 338/265.7 for 13C12 PCB126. In the MRM ion transition, the precursor ion represents the $M^{+\cdot}$ and the product ion represents either $[M\text{-}Cl]^+$ or $[M\text{-}2Cl]^+$. MRM transitions monitored with regard to hydroxy PCB metabolites: 340.8/340.9 for hydroxy PCB126 and 386.8/340.9 for dihydroxy PCB126. The precursor ion of the ion transition is a formic acid adduct: $[M+FA-H]^-$ and product ion is [M-H]-. All values were subsequently normalized for volume added and for IS recovery.

3.3.4 RNA isolation and polymerase chain reaction (PCR) amplification

Liver samples used to analyze oxidative stress and inflammatory mRNA markers were homogenized and mRNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. mRNA concentrations were then determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcription was performed using the AMV reverse transcription system (Promega, Madison, MI). The levels of mRNA expression were assessed by quantitative real-time PCR using a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems) as compared to constitutively expressed β-actin (forward primer: 5'-TGTCCACCTTCCAGCAGATGT-3'; reverse primer: 5'-GCTCAGTAACAGTCCGCCTAGAA-3') using the relative quantification method (ΔΔCt). Primer sequences (see Table 1) for SYBR Green reactions were designed using the Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

3.3.5. Immunoblotting assays

Liver samples used for protein analysis were homogenized in extraction RIPA buffer containing protease inhibitors (Pierce, Rockford, IL). Lysed tissue was centrifuged at 10,000 g for 30 min at 4 ºC followed by Bradford protein assay (Pierce). Protein samples were separated using 10% SDS-PAGE and subsequently were transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk buffer and incubated overnight at 4 ºC with the following primary antibodies: β-actin (product

#A2066, ~42 kD, Sigma, St. Louis, MO), GAPDH (product #sc-20357, ~37 kD, Santa Cruz Biotechnology, Dallas, TX), GSR (product #ab16801, ~58 kD, Abcam, Cambridge, MA), and NQO1 (product #ab34173, ~31 kD, Abcam). After washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and visualized using ECL detection reagents (Thermo, Waltham, MA).

Liver samples used for nuclear translocation assays were prepared and cytoplasmic and nuclear protein was extracted according to manufacturer protocol (NE-PER® Nuclear and Cytoplasmic Extraction Method, Thermo). Translocation samples subsequently were processed as seen above, and probed with the following primary antibodies: lamin (product #sc-7292, ~69 kD, Santa Cruz Biotechnology) and Nrf2 (product #sc-722, ~59 kD, Santa Cruz Biotechnology).

3.3.6. Data analyses

Statistical analysis was performed using Student's t-test for comparison of treatment groups. Overall, no statistical differences were exhibited between vehicle control groups, thus Student's t-test was used to analyze differences between PCB treatment groups. qRT-PCR mRNA analysis (n=8-10), Western blot protein analysis (n=8), and nuclear translocation analysis (n=4) represent three experimental replicates. A probability value of $p \leq 0.05$ was considered statistically significant. All statistical analyses were overseen by staff of the University of Kentucky Department of Statistics.

3.4. Results

3.4.1 Systemic toxicity associated with PCBs

PCB 126 levels in mouse plasma were examined as a measure of systemic PCB body burden and to determine green tea extract (GTE) diet supplementation's role in increasing PCB metabolism/excretion. Ion transitions of PCB 126 and OH-PCB 126 were measured by UFLC-MS/MS, normalized for sample volume, and compared to ion transitions of internal standard (13C12 PCB 126) with known concentration to determine PCB parent and metabolite concentrations (pmol/μL plasma). As seen in Fig. 3.2, plasma PCB 126 was found almost completely as its hydroxylated metabolite, OH-PCB 126, with concentrations of approx. 0.04 pmol PCB 126/μL plasma versus approx. 30 pmol OH-PCB 126/μL, respectively. GTE diet supplementation did not modulate PCB metabolism or plasma concentrations 24 h following PCB exposure, indicating that it plays a minimal role in pollutant clearance from the body. It is hypothesized, though, that

while GTE supplementation does not increase 24 h pollutant metabolism and clearance, it prepares the body's defense mechanisms to more effectively modulate oxidative stress present during toxicant acute insult.

3.4.2 F_2-isoprostane levels are significantly reduced in green tea extract-supplemented, PCB-exposed mice

Analysis of F_2-isoprostanes (F_2-IsoPs), prostaglandin-like eicosanoids formed during fatty acid peroxidation, has emerged as the most reliable method for assessing *in vivo* oxidative stress[187]. Plasma samples from mice fed control and GTE-supplemented diets and subsequently treated with vehicle or PCB 126 (n=8-10) were analyzed to determine GTE's role in modulating environmental toxicant-induced oxidative stress. Plasma F_2-IsoP (including PGF2α, 8-iso-PGF2α, iPF2α-III, 8-epiPGF2α, 8-isoprostane, and 15-F2t isoprostanes) and F_2-IsoP metabolite (13,14-dihydro-15-ketoPGF2α) concentrations were determined using UFLC-MS/MS and integrating peak area (area under the curve, AUC) with regard to known internal standard concentrations (AUC/IS). As seen in Fig. 3.3, GTE diet supplementation led to drastically decreased F_2-IsoP levels ($p<0.05$) in mice treated with PCB 126, indicating that GTE acts as a strong antioxidant to modulate against environmental toxicant insult. Additionally, GTE drastically decreased PCB-induced F_2-IsoP metabolite production ($p<0.05$); F_2-IsoP metabolite analysis is developing as an even more sensitive measure of *in vivo* oxidative stress because the metabolites do not undergo autoxidation and artificial production as has been seen with parent F_2-IsoP[188]. Interestingly, GTE supplementation led to no significant modulation of F_2-IsoP parent or metabolite levels under control situations, indicating that antioxidant modulation occurs primarily when a system is under stress.

3.4.3. Green tea extract increases mRNA antioxidant response

Antioxidant enzyme levels were measured in mouse liver to further develop the role of GTE diet supplementation in modulating environmental insults *in vivo.* Table 3.2 highlights antioxidant mRNA markers tested and overall results. qRT-PCR analysis (n=8-10) shows a significant upregulation in catalase, glutathione peroxidase (Gpx3), glutaredoxin (Grx2), glutathione reductase (GSR), glutathione S-transferases (GSTa1, GSTa4, GSTm1, GSTm2, and GSTm3), NAD(P)H dehydrogenase [quinone] 1 (NQO1), superoxide dismutase 1 (SOD1), thioredoxin 2 (Trx2), and thioredoxin reductase 1 (TrxR1) during the concomitant treatment of PCB 126 and GTE diet supplementation.

As before, in most cases GTE supplementation did not significantly modulate antioxidant response without the presence of secondary external insult. Analysis of NQO1 and GSTm3, enzymes associated with detoxification, exhibited significantly increased mRNA levels above vehicle control diet levels in the presence of PCB 126, while GTE diet supplementation drastically induced antioxidant mRNA expression following PCB insult. mRNA levels of SOD1, critical for modulating harmful superoxide radicals produced during toxicant insult, were significantly decreased following PCB gavage, while GTE supplementation returned mRNA expression to vehicle control diet levels. While PCB insult did not modulate GSR (an important cellular antioxidant) mRNA levels in mice fed vehicle control diets, GTE diet supplementation led to a significantly increased antioxidant response (see Fig. 3.4, $p<0.01$). Additionally, data shown in Fig. 3.9 further expounds upon these trends in response to GTE supplementation. For example, thioredoxin 2 (Trx2, an important redox protein) mRNA levels are significantly upregulated in the concomitant presence of GTE and PCB 126 although GTE does not induce increased antioxidant activity without the addition of secondary external insult.

3.4.4 Green tea extract increases NQO1 and GSR antioxidant protein response against PCB 126

Antioxidant marker protein analysis was performed in order to better understand the role that GTE diet supplementation plays in increasing the body's defensive mechanisms against toxicant insult. Proteins of interest were compared to multiple housekeeping genes, β-actin and GAPDH, to further substantiate findings. In PCB 126-treated mouse liver samples, NQO1 protein was significantly upregulated when fed a GTE supplemented diet, as shown in Fig. 3.5 when quantified against GAPDH ($p<0.01$). The associated representative blot continues trends seen in mRNA with a large increase in antioxidant protein activity in GTE supplemented mice exposed to PCB insult. GSR protein activity was also statistically increased in response to diet supplementation and continues the trend seen in mRNA analysis in which neither GTE supplementation nor PCB treatment led to modulation of antioxidant response while their concomitant treatment led to significant upregulation ($p\leq0.05$).

3.4.5. Green tea extract drives Nrf2 nuclear translocation in the presence of PCB 126

Nuclear translocation assays are commonly used techniques that serve as a representation of cellular transcriptional activation. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) nuclear translocation was analyzed in comparison to lamin, a nuclear fraction housekeeping gene; liver samples from GTE-supplemented mice exposed to PCB 126 showed a trend toward increased nuclear abundance of Nrf2 ($p=0.1$, $n=4$). As in antioxidant mRNA and protein studies, GTE without a concomitant insult from PCB did not modulate Nrf2 activity and translocation. The Nrf2 antioxidant pathway plays a pivotal role in modulating oxidative stress, and therefore its upregulation by GTE diet supplementation, as seen in Fig. 3.6, is an important indicator of GTE's ability to increase the body's responsiveness toward environmental stressors.

3.4.6 Green tea extract modulates inflammatory and xenobiotic-related markers in the presence of PCB 126

Cytochrome P450 (e.g., CYP1A1 and CYP1B1), a family of enzymes controlled by the aryl hydrocarbon receptor (AhR) and Nrf2 proteins that are vital for the metabolism of xenobiotic substances, including toxicants, was analyzed from liver samples to determine potential modulation of its activity in response to GTE diet supplementation. Substantial CYP upregulation was seen in the presence of PCB insult, as has been shown in past literature, while concurrent GTE supplementation causes a significant upregulation in CYP1B1 mRNA analysis (as seen in Fig. 3.7), indicating potential for higher toxicant degradation and clearance. In mice fed GTE-supplemented diets and exposed to PCB, major indicators of inflammation including monocyte chemoattractant protein-1 (MCP-1, also referred to as chemokine (C-C motif) ligand 2, CCL2) and CCL3 (also referred to as macrophage inflammatory protein-1α, MIP-1α) were significantly decreased back to vehicle control levels. Interestingly, GTE supplementation alone led to significant increases in inflammatory marker mRNA levels, although these levels returned to vehicle control levels with concomitant treatment ($p<0.05$).

3.4.7. The AhR is implicated as a control mechanism both in PCB 126 toxicity and antioxidant response

As seen in Fig. 3.8, GTE diet supplementation led to significant upregulation of AhR mRNA levels in liver in the presence of PCB 126, a dioxin-like AhR ligand. PCB 126 insult did not induce AhR mRNA in vehicle control, vehicle + GTE control, and PCB control settings, but, interestingly, concomitant treatment led to a two-fold upregulation,

similarly to that seen in mRNA and protein antioxidant and inflammatory markers, thus allowing for increased *in vivo* toxicant clearance ($p<0.01$). Nrf2 mRNA levels were also significantly increased during PCB 126 insult, although GTE supplementation did not cause significant modulation of PCB toxicity. AhR and Nrf2 signaling pathways control both xenobiotic responses and inflammatory cascades, therefore their modulation by GTE diet supplementation implicates further GTE's role in strengthening antioxidant response toward insult by environmental pollutants.

3.5. Discussion

Healthy nutrition can positively influence, or lessen, the human health risks associated with exposure to mixtures of environmental chemicals[189]. The liver is one tissue particularly vulnerable to environmental pollutants, and especially PCB-induced toxicity [173]. One type of liver disease that affects more than 20% of Americans is nonalcoholic fatty liver disease, which can lead to nonalcoholic steatohepatitis[190]. Industrial toxicants also have been linked to secondary insults that can lead to steatohepatitis[190]. Mechanisms defining the involvement of environmental pollutants in the pathology of liver diseases may include a compromised redox status or toxicant-induced increase in oxidative stress.

Lifestyle modifications that include healthful nutrition have been suggested as a powerful means of reducing the vulnerability to environmental insults[189] and in reducing the risk to environmental toxicant-induced liver disease[190]. For example, green tea extract has been shown to protect against hepatic steatosis in obese mice by reducing oxidative stress and enhancing hepatic antioxidant defenses[191]. In the present study we observed significant protection against PCB 126-induced oxidative stress by dietary supplementation with green tea extract. In fact, mice supplemented with green tea extract and subsequently exposed to PCB displayed a decrease in overall F_2-isoprostane and metabolite levels compared to animals on a control diet exposed to PCB. We did not see a large increase in oxidative stress as evidenced by an increase in F_2-isoprostane levels in mice fed a control diet and subsequently gavaged with PCB. This may have been due to the fact that the amount of administered PCB 126 reflects more physiologically relevant PCB concentrations in humans than previous studies using high concentrations of PCB congeners (e.g., 150 μmol/kg versus 5 μmol/kg used herein)[192]. We hypothesize that levels of PCBs encountered by humans today initiate low levels of chronic oxidative stress and inflammation that, together with multiple other

factors including poor diet and exposures to other environmental stressors, leads to augmented or exacerbated human disease. The protective properties of green tea have been studied extensively, and recent human studies suggest that consumption of green tea may protect against cardiovascular disease and some forms of cancer, have anti-hypertensive and anti-obesity effects, and contribute to antibacterial and antiviral activity[193].

Certain environmental pollutants, and in particular ligands for the AhR, induce oxidative stress, in part via induction and uncoupling of cytochrome P450 enzymes[20a]. Hepatic changes in lipid composition, altered membrane structure and membrane functions are well-described phenomena of PCB-induced liver damage[194]. The protective properties of green tea extracts against PCB-induced pathologies, and in particular liver damage, may be numerous. For example, green tea may normalize the formation of lipid peroxide products induced by exposure to toxicants to prevent hepatic fibrosis[185], or green tea may favorably regulate intestinal tight junction proteins or overall intestinal barrier function[195]. It has been proposed that green tea can inhibit the intestinal absorption of lipids and highly lipophilic organic compounds[130]. Induction of antioxidant enzymes by green tea also may contribute to its tissue protective properties[196]. Our current study support this concept, and we observed an increased induction of antioxidant genes such as SOD1, GSR, NQO1 and GST in mice that were fed a green tea-supplemented diet and subsequently challenged with PCB, compared to animals exposed to the control diet plus PCB. This may explain in part the observed decrease in overall oxidative stress due to green tea supplementation. Of particular interest is our observed induction of NQO1, a Nrf2 target gene, which suggests that green tea protects in part by modulation of the Nrf2/ARE pathway. In fact, it has been shown that food polyphenols, including the green tea polyphenol EGCG, the primary component of GTE tested herein, can modulate Nrf2-mediated antioxidant and detoxifying enzyme induction[197]. Our own work with vascular endothelial cells further suggests that multiple pathways including lipid raft caveolae and the antioxidant defense controller Nrf2 play a role in nutritional modulation of PCB-induced vascular toxicity[13b] and that cross-talk between caveolae-related proteins and cellular Nrf2 may be required for optimal cytoprotection by green tea catechins and other diet-derived polyphenols.

Many groups, including ours, have shown that green tea catechins such as EGCG can upregulate basal levels of antioxidant enzymes *in vitro*[116b, 193, 198]. Interestingly, our overall results show that green tea extract supplemented in the diet acts as an

antioxidant only in the presence of a secondary stressor, in this case, the pro-inflammatory coplanar PCB 126. The inconsistencies between *in vitro* and *in vivo* studies may be explained by the relatively high doses of tea catechins usually employed in cell culture or the fact that most tea catechins are quickly biotransformed *in vivo* to metabolites that exhibit differential physiological effects[196]. There are many other examples of instances where supplementation with GTE or specific catechins is protective in *in vivo* models of inflammation and oxidative stress. For many of our investigated antioxidant enzymes we saw decreased expression in the presence of PCB when fed a control diet, but levels were upregulated, many returning to control vehicle levels in PCB groups fed a GTE-rich diet (e.g., SOD1 in Fig. 3.4). These observations are in line with other groups who investigated GTE effects on other stressors including ethanol toxicity and bacterial infection[199].

Our past work in cell culture points to the antioxidant controller Nrf2 as a major player in nutritional modulation of PCB toxicity. Many nutrients other than green tea catechins, including resveratrol, found in the skins of grapes, and sulforaphane, which is found in broccoli, have been shown to activate Nrf2[123c, 137]. Nrf2 can become transcriptionally active through multiple mechanisms including direct phosphorylation by PKC delta and loss of contact between Nrf2 and inhibitory kelch-like ECH-associated protein 1 (Keap1)[99b]. Upon activation, Nrf2 is able to evade ubiquitination, enter the nucleus, and bind *cis*-acting antioxidant response elements in target genes such as NQO1[82a]. Nrf2 activation leads to decreased overall oxidative stress and inflammation, which is a hallmark of PCB toxicity[100]. In this work we observed a relatively significant trend toward increased NRF2 translocation to the nucleus in animals supplemented with GTE and subsequently exposed to PCB (Fig. 3.6). More interestingly and novel, we also saw a drastic increase in AhR mRNA expression in this same treatment group (Fig. 3.8). This upregulation was mirrored in increases in both CYP1A1 and CYP1B1 mRNA levels in mice fed a GTE rich diet and subsequently exposed to PCB. An increase in AhR may help to detoxify the acute exposure to PCB by increasing metabolism-assisted excretion. Although a consistent, steady upregulation of AhR may create a negatively imbalanced redox situation, the GTE's ability to upregulate AhR only in the presence of a toxicant may in some cases be a protective and positive mechanism. Other groups have shown that different catechins within GTE display either antagonistic or agonist activities against CYP1A1[200], but to our knowledge no group has reported the mRNA upregulation as seen in Fig. 3.7. In our analysis of PCB concentrations in plasma we observed a very

slight trend towards decreased levels of parent PCB 126 in the plasma of mice supplemented with GTE (Fig. 3.2). Although plasma levels may be a good overall picture of body-burden of PCBs, in the future, collecting urine and feces may paint a clearer picture of GTE's involvement with detoxification and excretion. Also, we may have been able to see a more significant decrease in PCB levels and or a modulation of PCB hydroxy metabolite in mice supplemented with GTE if we sacrificed the mice more than 24 hours after the final PCB dose[130a].

PCBs can induce vascular inflammation by upregulating pro-inflammatory mediators such as MCP-1 and CCL3. We hypothesized that GTE would downregulate basal levels of these inflammatory markers in vehicle treated mice as well as decrease PCB-mediated upregulation in PCB 126 treated mice. Interestingly however, for both of our inflammatory markers we saw a significant increase in mRNA levels in vehicle treated mice supplemented with GTE (Fig. 3.7). This observation would suggest that the dose of GTE used in this study may not be optimal, and perhaps toxic to some degree, in basal levels of oxidative stress and inflammation. Other groups have shown GTE toxicities at certain doses *in vivo*[201], and interestingly, data illustrating protection seems to be more conclusive in animal models of oxidative stress and inflammation. Importantly, for our study, both MCP-1 and CCL3 mRNA levels return to vehicle treated control diet levels in mice fed GTE and subsequently exposed to PCB. This may point to GTE as exhibiting possible *hormetic* activity by inducing a slight response by the organism that ultimately primes the protective antioxidant system for a future stressor, i.e., Superfund pollutant exposure. Understanding hormesis and the role that nutrients can play is an extremely interesting scientific discipline and demands much more future investigation[202].

In summary, our current study supports our *in vitro* data that green tea catechins can protect against PCB 126-induced cytotoxicity by reducing oxidative stress[73b]. Our current *in vivo* data contributes to the overall hypothesis that nutrition can modulate environmental insults. More studies are needed to further understand detailed mechanisms of protective benefits to consume diets high in protective and healthful nutrients such as plant-derived polyphenols and other bioactive compounds.

Table 3.1. Primers used for qRT-PCR

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'	Fragment size
Inflammatory and xenobiotic-related markers			
CCL3	CACCCTCTGTCACCTGCTCAA	TGGCGCTGAGAAGACTTGGT	100 bp
CYP1A1	TGGAGCTTCCCCGATCCT	CATACATGGAAGGCATGATCTAGGT	100 bp
CYP1B1	TGCATCGGTGAGGAACTGTCT	CTCATGTTTGAGGACTCATTTTGG	104 bp
MCP-1	GCAGTTAACGCCCCACTCA	CCTACTCATTGGGATCATCTTGCT	63 bp
Antioxidant markers			
AhR	GACCAAACACAAGCTAGACTTCACACC	CAAGAAGCCGGAAAACTGTCATGC	200 bp
Catalase	CAGAGAGCGGATTCCTGAGAGA	CTTTGCCTTGGAGTATCTGGTGAT	100 bp
Gpx2	GTGGCGTCACTCTGAGGAACA	CAGTTCTCCTGATGTCCGAACTG	125 bp
Gpx3	CATACCGGTTATGCGCTGGTA	CCTGCCGCCTCATGTAAGAC	80 bp
Grx2	CATCCTGCTCTTACTGTTCCATGGCCAA	TCATCTTGTGAAGCGCATCTTGAAACTGG	123 bp
GSR	TCGGAATTCATGCACGATCA	GGCTCACATAGGCATCCCTTT	100 bp
GSTa1	AAGCCCGTGCTTCACTACTTC	GGGCACTTGGTCAAACATCAAA	159 bp
GSTa4	TACCTCGCTGCCAAGTACAAC	GAGCCACGGCAATCATCATCA	109 bp
GSTm1	ATACTGGGATACTGGAACGTCC	AGTCAGGGTTGTAACAGAGCAT	349 bp
GSTm2	ACACCCGCATACAGTTGGC	TGCTTGCCCAGAAACTCAGAG	118 bp
GSTm3	CCCCAACTTTGACCGAAGC	GGTGTCCATAACTTGGTTCTCCA	208 bp
NQO1	GGCATCCAGTCCTCCATCAA	GTTAGTCCCTCGGCCATTGTT	100 bp
Nrf2	GAGTCGCTTGCCCTGGATATC	TCATGGCTGCCTCCAGAGAA	100 bp
SOD1	GAAACAAGATGACTTGGGCAAAG	TTACTGCGCAATCCCAATCA	100 bp
Trx2	GCTAGAGAAGATGGTCGCCAAGCAGCA	TCCTCGTCCTTGATCCCCACAAACTTG	168 bp
TrxR1	GGCCAAAATCGGTGAACACATGGAAG	CGCCAGCAACACTGTGTTAAATTCGCCCT	175 bp

Table 3.2. The effect of green tea extract (GTE) diet supplementation on PCB 126-induced mRNA inflammatory, xenobiotic-related, and antioxidant markers.

	5 µmol PCB 126/kg mouse (fold change)		
Gene name	Control diet	Control + 1% GTE	p-value
Inflammatory and xenobiotic-related markers			
CCL3	2.051±0.224	0.945±0.116	<0.001
CYP1A1	915.208±136.510	1169.338±78.900	N.S.
CYP1B1	87.504±7.694	146.998±12.329	<0.001
MCP-1	1.909±0.478	0.745±0.235	0.012
Antioxidant markers			
AhR	0.771±0.096	1.506±0.131	<0.001
Catalase	0.495±0.057	0.817±0.071	0.003
Gpx2	0.339±0.161	0.682±0.098	0.08
Gpx3	0.925±0.167	1.411±0.159	0.004
Grx2	0.213±0.015	0.486±0.078	0.003
GSR	0.702±0.074	1.245±0.097	0.001
GSTa1	14.771±2.911	22.955±3.975	0.034
GSTa4	0.896±0.117	2.222±0.245	<0.001
GSTm1	2.306±0.450	4.024±0.301	0.006
GSTm2	0.868±0.097	1.366±0.114	0.004
GSTm3	4.469±0.664	18.596±1.819	<0.001
NQO1	1.980±0.051	6.138±0.031	0.006
Nrf2	3.088±0.307	3.212±0.234	N.S.
SOD1	0.311±0.041	0.684±0.063	<0.001
Trx2	0.731±0.050	1.556±0.152	<0.001
TrxR1	1.522±0.143	2.673±0.276	0.002

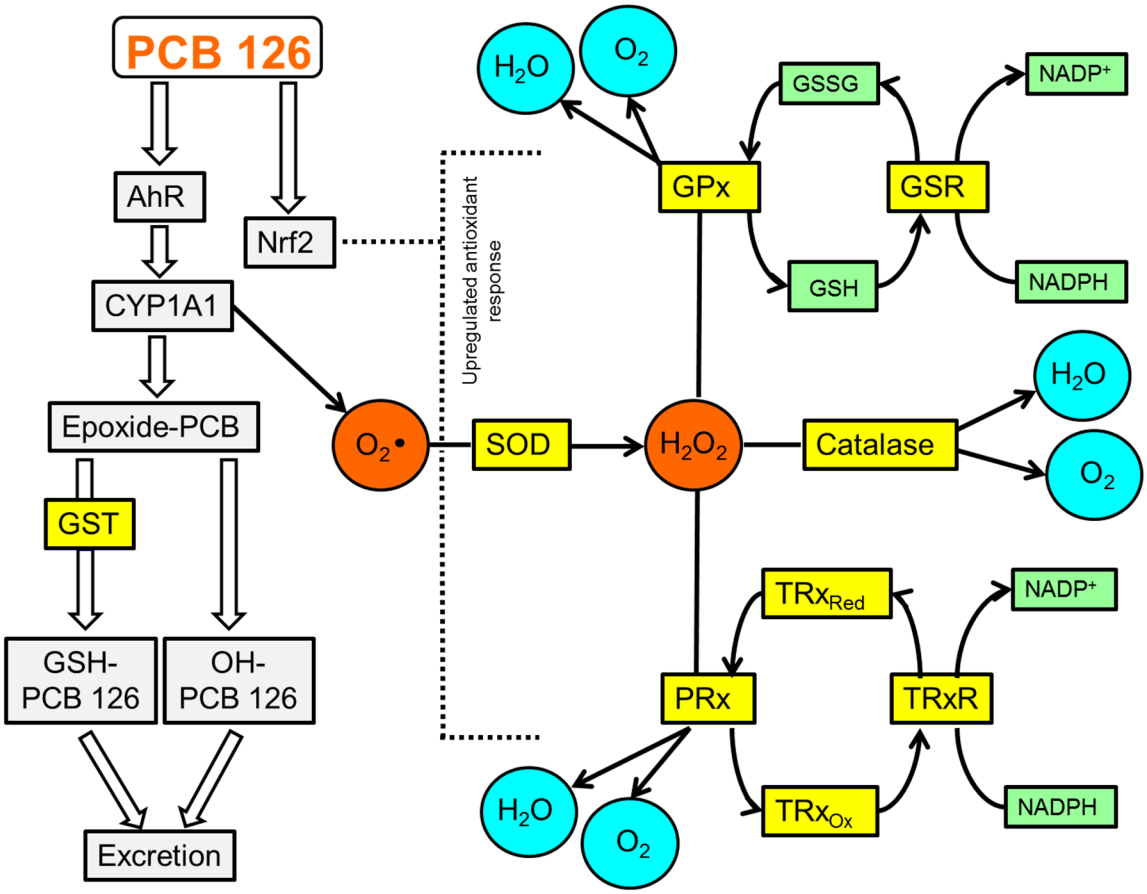

Fig. 3.1. Proposed signaling pathway for PCB detoxification *in vivo*. PCB 126, an AHR ligand and activator of NRF2, causes CYP1A1 upregulation, which leads to superoxide production. Green tea extract (GTE) diet supplementation effectively upregulates redox-related enzymes in the presence of PCB 126 which allows for a more efficient antioxidant response to environmental insult.

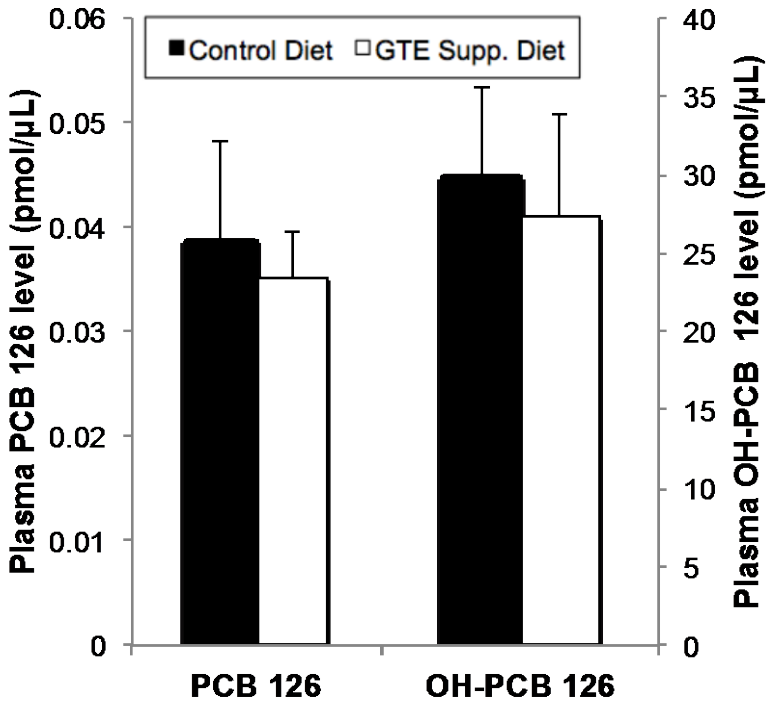

Fig. 3.2. The effect of green tea extract (GTE) diet supplementation on systemic PCB 126 concentration and metabolism. PCB 126 and its hydroxy metabolites were measured in mouse plasma by UFLC/MS MS and normalized to sample volume and internal standard recovery. PCB 126 is heavily metabolized *in vivo*, as seen by very low levels of parent PCB 126 remaining in plasma samples while its hydroxylated metabolites predominate. GTE supplementation did not significantly modulate systemic PCB or metabolite concentrations. Data are presented as mean±S.E.M (n=5).

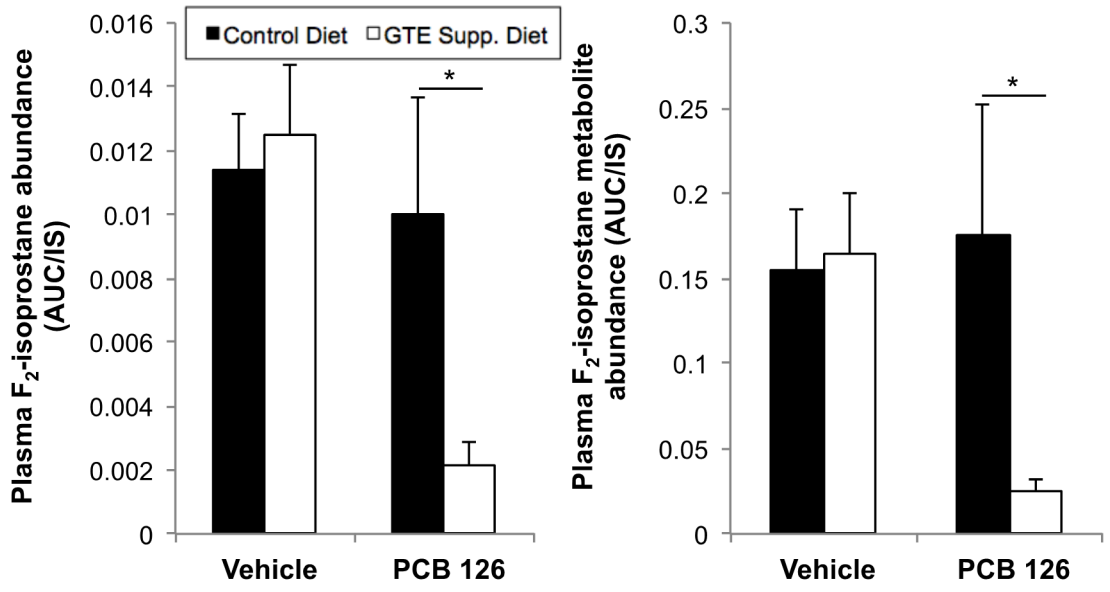

Fig.3.3. PCB 126-induced oxidative stress is modulated by green tea extract (GTE) diet supplementation. Plasma F_2-isoprostane and metabolite levels were measured by HPLC/MS MS to assess *in vivo* oxidative stress induced by PCB 126 that is potentially mitigated by GTE supplementation. Relative levels of combined F-2 IsoPs, including PGF2α, 8-iso-PGF2α, iPF2α-III, 8-epiPGF2α, 8-isoprostane, and 15-F2t isoprostanes, were determined by averaging the AUC integration values from retention times of 8 and 11.3 minutes (Q1 = 353.144, Q2 =193.1). Additionally, the level of 13,14-dihydro-15-ketoPGF2α, an F-2 IsoP metabolite, was determined by integrating its peak at 11.3 minutes (Q1 = 355.2, Q2 = 311.4). Data are presented as mean±S.E.M. (n=8-10). GTE supplementation significantly decreased oxidative stress induced by PCB 126 treatment (*$p<0.01$).

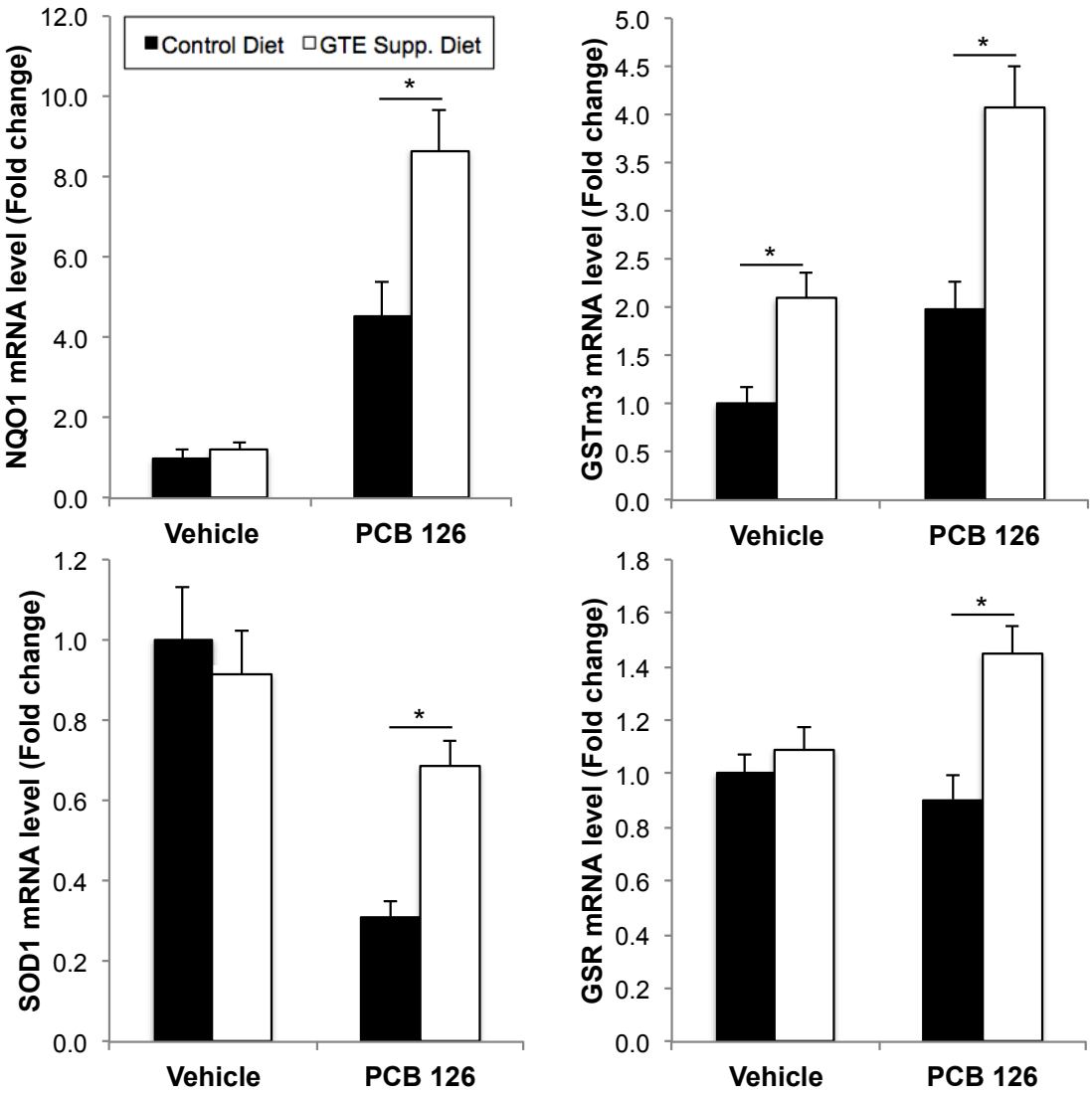

Fig. 3.4 Relative mRNA levels of representative antioxidant enzyme markers. Overall GTE supplementation did not significantly increase antioxidant mRNA levels in control diets, but, in the presence of environmental perturbation (i.e. PCB 126 gavage), significantly higher antioxidant levels were seen in mouse liver above non-supplemented diet. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M (*$p<0.01$, n=8-10). See Table 3.2 and Fig. 3.9. for more information concerning all antioxidant markers tested.

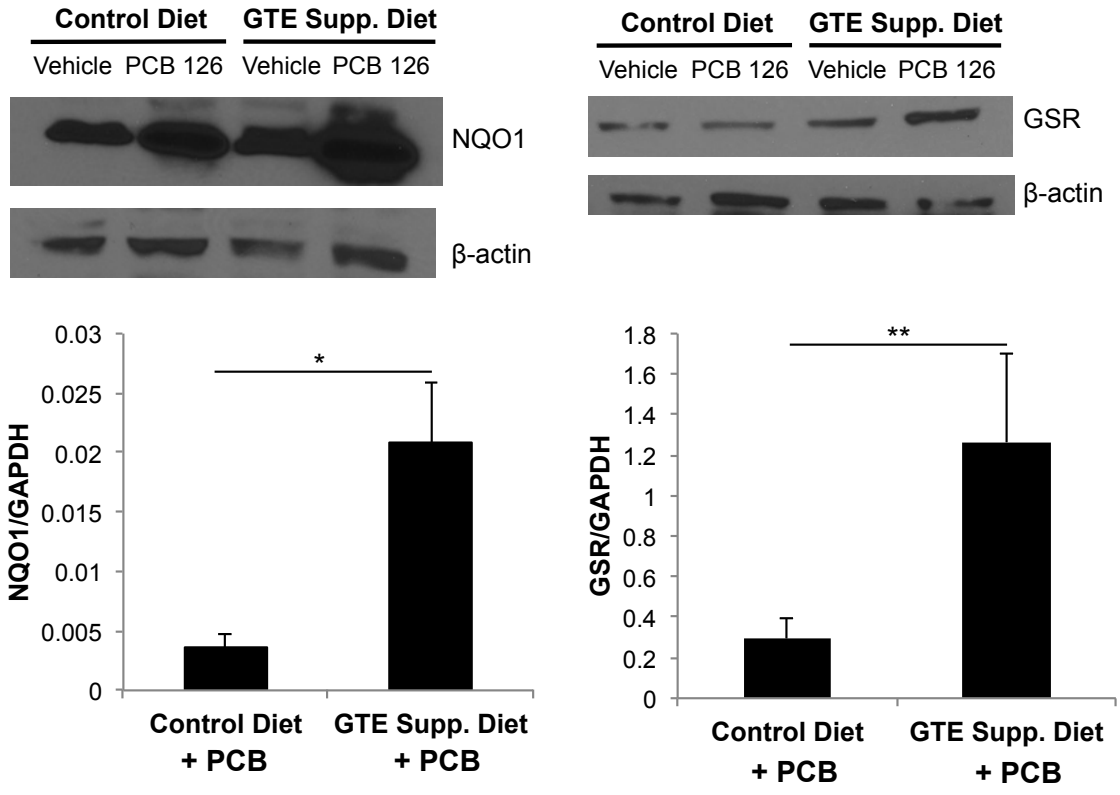

Fig. 3.5. GTE supplementation leads to increased antioxidant protein expression in the presence of PCB 126. Protein samples were separated through gel electrophoresis and probed with NQO1 and GSR primary antioxidant-related antibodies. Statistically significant increases in antioxidant protein activity were seen in PCB 126-treated mice that were fed a GTE-supplemented diet. In addition to visualized Western blot comparison to β-actin, samples were compared to GAPDH for densitometry quantification to further substantiate findings. GTE supplemented mice exposed to PCB showed a significant increase in protein expression, indicating a strengthened antioxidant response due to GTE supplementation (*$p<0.01$,**$p\leq0.05$, N=8).

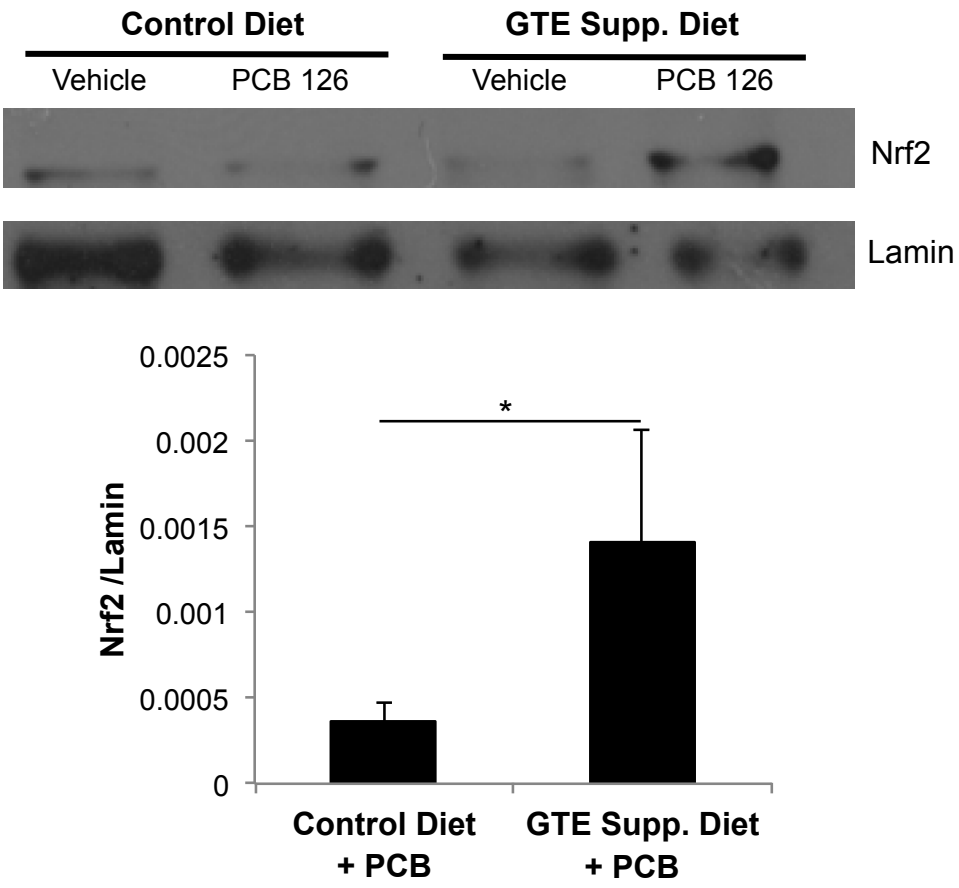

Fig. 3.6. Mice fed a 1% GTE-supplemented diet and subsequently exposed to PCB 126 displayed increased Nrf2 activation, as evidenced by increased Nrf2 translocation to the nucleus, compared to mice fed 10% fat control diet and exposed to PCB. Lamin was used as a nuclear fraction housekeeping gene for densitometry quantifications. GTE supplemented mice exposed to PCB showed a trend toward increased nuclear abundance of Nrf2 (*p=0.1, n=4).

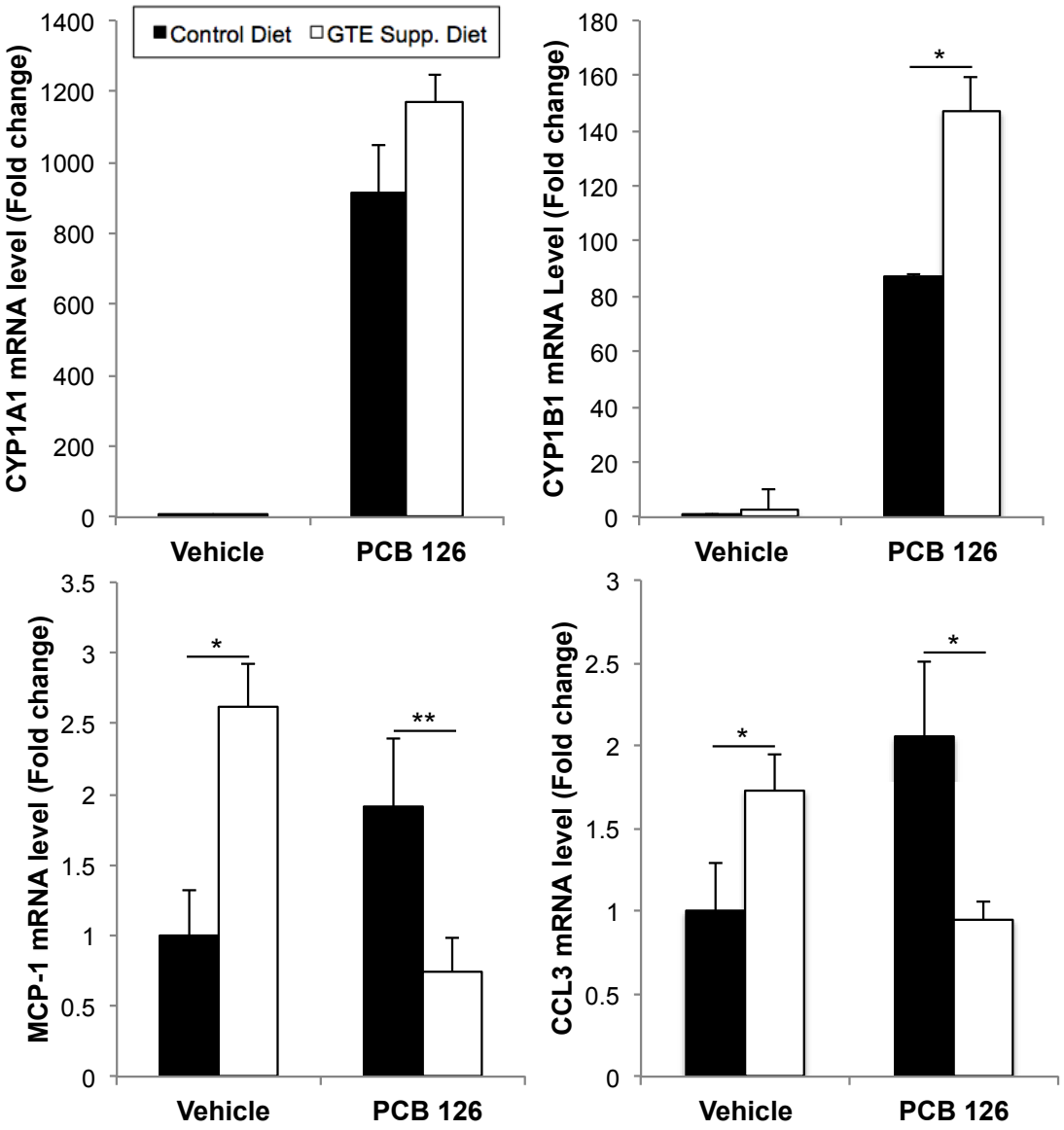

Fig. 3.7. Relative mRNA levels of inflammatory and xenobiotic-related markers. GTE supplementation led to increased cytochrome P450 (CYP1A1 and CYP1B1) mRNA expression in the presence of both GTE and environmental toxicant (i.e., PCB 126), indicating increased activity for toxicant degradation and/or excretion. MCP-1 and CCL3 inflammatory markers were statistically increased in GTE-supplemented mice liver samples but toxicant-induced inflammatory markers returned to control levels due to GTE supplementation. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M. (*$p<0.01$, **$p<0.05$, n=8-10).

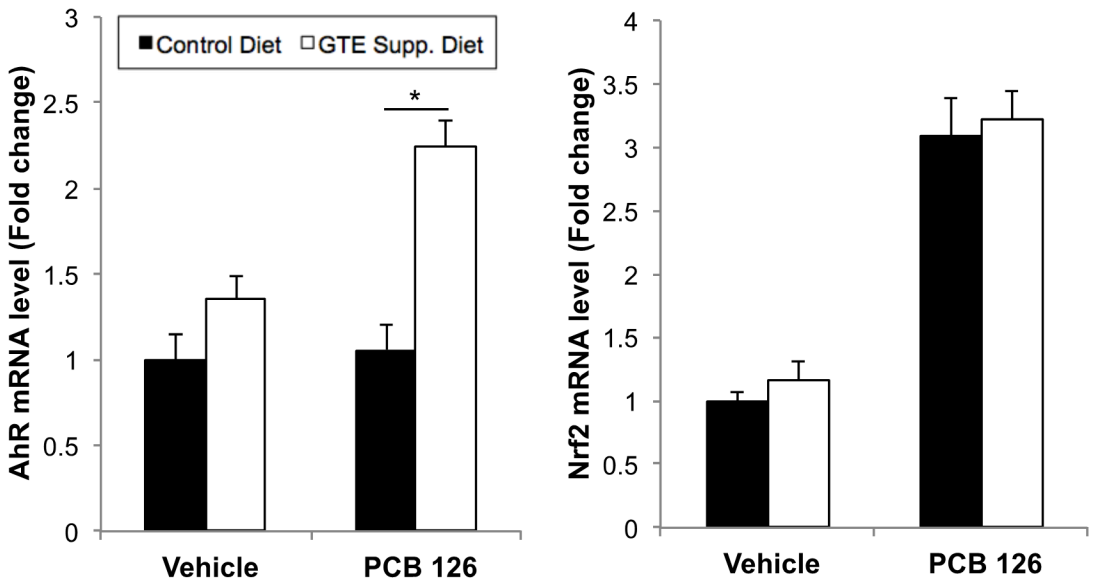

Fig. 3.8. GTE diet supplementation leads to significant upregulation of AhR mRNA levels in the presence of PCB 126, thus increasing *in vivo* toxicant clearance. Nrf2 mRNA levels are significantly increased during PCB 126 insult, although GTE supplementation does not cause statistically significant modulation of PCB toxicity. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Data are presented as mean±S.E.M (n=8-10). GTE supplementation significantly increased AhR in the presence of PCB 126 treatment (*p<0.01).

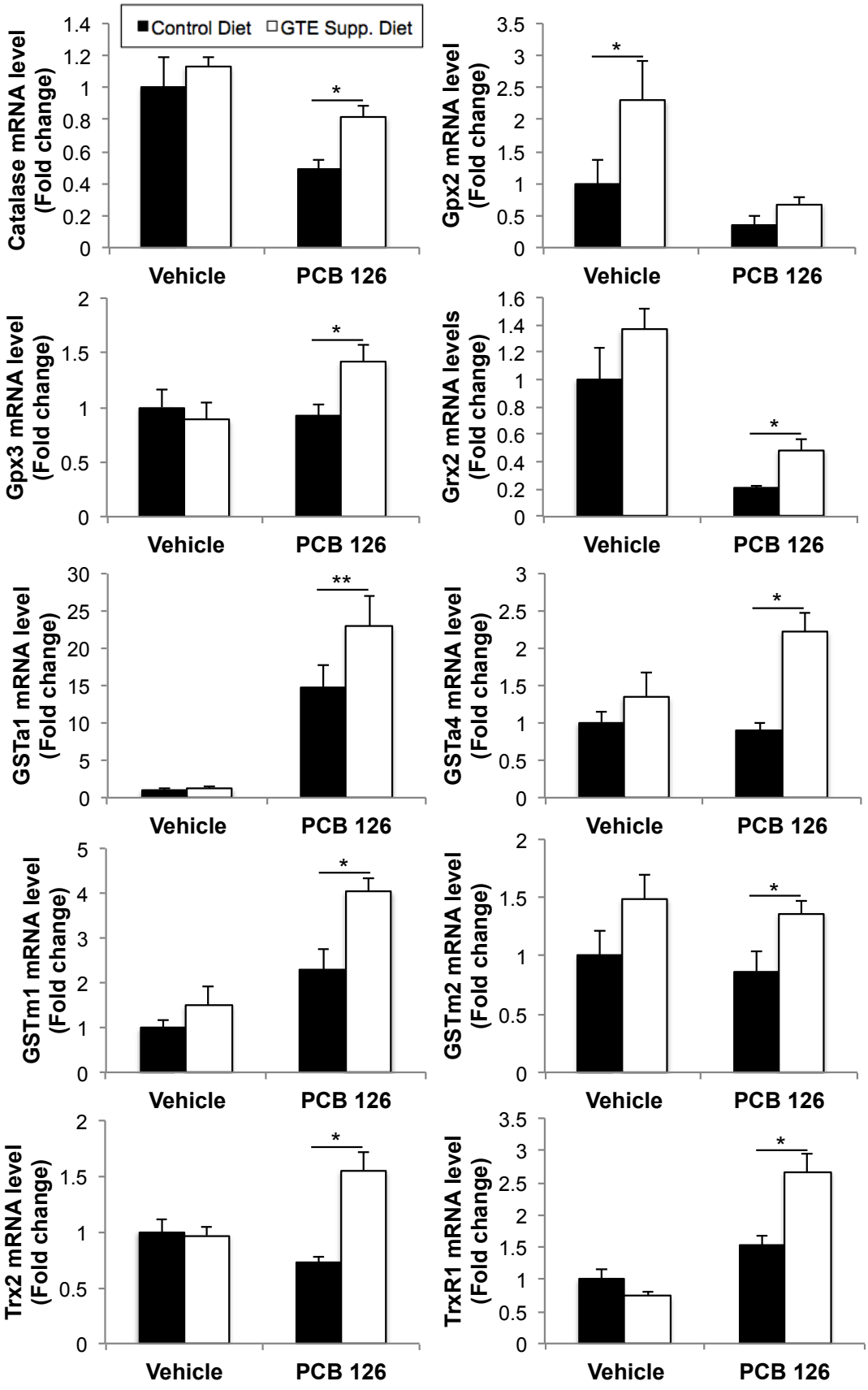
Control Diet
GTE Supp. Diet
Catalase mRNA level (Fold change)
Gpx2 mRNA level (Fold change)
Gpx3 mRNA level (Fold change)
Grx2 mRNA levels (Fold change)
GSTa1 mRNA level (Fold change)
GSTa4 mRNA level (Fold change)
GSTm1 mRNA level (Fold change)
GSTm2 mRNA level (Fold change)
Trx2 mRNA level (Fold change)
TrxR1 mRNA level (Fold change)
Vehicle
PCB 126

Fig. 3.9. Relative mRNA levels of antioxidant enzyme markers. Overall, GTE supplementation did not significantly increase antioxidant mRNA levels in control diets, but, in the presence of environmental perturbation (i.e., PCB 126 gavage), significantly higher antioxidant levels were seen in mouse liver above non-supplemented diet. All values were determined using the relative quantification method (ΔΔCt) as a fold change from control. Vehicle and PCB 126-treated mice with 10% fat control and GTE-supplemented diets: n=8-10. Data are presented as mean±S.E.M (*$p<0.01$, **$p<0.05$). See Table 3.2 and Fig. 3.4. for more information concerning antioxidant markers tested.

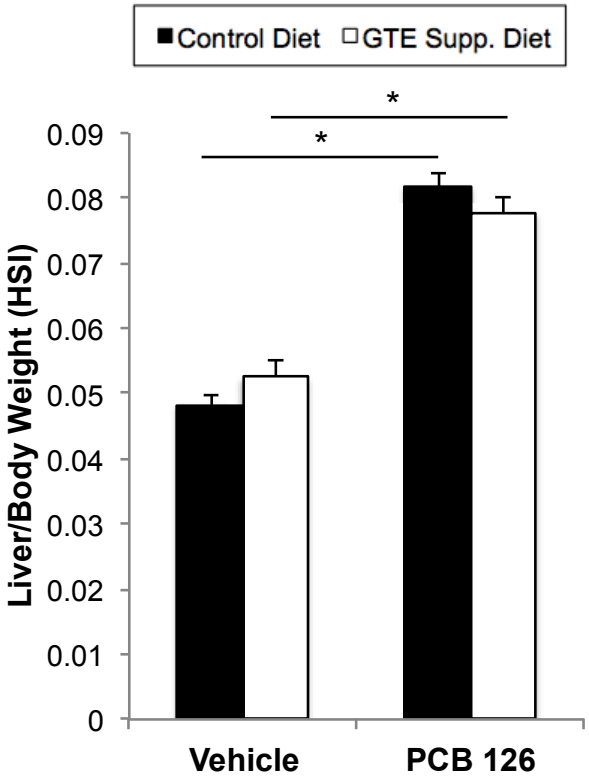

Fig. 3.10. PCB 126 exposure leads to a significant increase in liver/body weight ratio (hepatosomatic index, HSI) in both control and GTE-supplemented diets, although GTE supplementation did not significantly mitigate this PCB-induced increase. Statistical analysis was performed using two-way ANOVA followed by Tukey test with *$p<0.001$. A trend toward a statistically significant interaction between diet and PCB 126 treatment ($p=0.06$) was also seen.

Table 3.3. A: Dietary make up of low fat control and low fat 1% green tea extract mouse diets. B: Polyphenol formulation of Taiyo 1% GTE.

A:

	1% GTE-supplemented diet		**Control diet**	
	g/kg	*kcal*	g/kg	*kcal*
Protein	19 *(20%)*		19 *(20%)*	
Carbohydrate	67 *(70%)*		67 *(70%)*	
Fat	4 *(10%)*		4 *(10%)*	
Casein	190	*800*	190	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	299	*1260*	299	*1260*
Maltodextrin 10	33	*140*	33	*140*
Sucrose	332	*1400*	332	*1400*
Cellulose, BW200	47	*0*	47	*0*
Soybean Oil	24	*225*	24	*225*
Lard	19	*180*	19	*180*
Mineral Mix S10026	9	*0*	9	*0*
Vitamin Mix V10001	9	*40*	9	*40*
Green Tea Extract	10	--	0	--
Total	1065	*4057*	1055	*4057*

B:

Manufacturer's Description: Certified organic, excellent tea taste, stable in beverages.
Catechins>15%(ECG average 11.75%), caffeine<10% (average 6.6%)

	% Total	**%Catechins**
Caffeine	**7.5**	
EGC	**9.3**	**35.1**
EC	**2.8**	**10.6**
EGCG	**11.7**	**44.1**
ECG	**2.7**	**10.2**
Total Catechins	**26.6**	**100.0**

Chapter 4. Nitration of linoleic acid modulates polychlorinated biphenyl-induced endothelial cell dysfunction

4.1 Synposis

Data now implicate correlations between persistent organic pollutants, such as polychlorinated biphenyls, and cardiovascular diseases. We have shown that coplanar PCBs can initiate endothelial cell dysfunction, oxidative stress, and inflammation. Recent evidence shows that toxicant-nutrient interactions exist, and a diet high in pro-inflammatory omega-6 lipids such as linoleic acid, may exacerbate PCB-induced cardiovascular disease. However, recently discovered anti-inflammatory lipid metabolites, such as nitro-fatty acids, may limit this exacerbated effect. The aim of our research was to determine if pro-inflammatory lipids could increase the toxicity of the coplanar PCB 126, and if nitration of these pro-inflammatory lipids could ameliorate this exacerbated effect. First, vascular endothelial cells were treated with linoleic acid or nitrolinoleic acid and harvested for mRNA. Linoleic acid treatment alone increased AhR, CYP1A1, MCP-1, and VCAM-1 message in a dose dependent manner, but the upregulation of these pro-inflammatory mediators was not observed in nitrolinoleic treated cells. Also, nitrolinoleic acid upregulated heme-oxygenase 1, a protective antioxidant enzyme, which may help to explain the anti-inflammatory role of nitro-fatty acids. Next, vascular endothelial cells were pretreated with linoleic acid or nitrolinoleic acid and subsequently exposed to physiologically relevant concentrations of PCB 126. Treatment with linoleic acid was pro-inflammatory as evidenced by increased mRNA levels (RT-PCR) of vascular cellular adhesion molecular-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) and caveolin-1 (Cav-1). Levels of MCP-1 and VCAM-1 were most exacerbated in linoleic acid/PCB treated cells. Interestingly, the addition of a nitro group to linoleic acid prevented this exacerbated PCB effect. More research is necessary to determine if the protective effects observed herein are due to an upregulated antioxidant defense or rather the nitro group protecting against oxidation and subsequent formation of toxic pro-inflammatory metabolites. Understanding diet toxicant interactions is a critical step towards more effective risk assessment and public health outcomes for at-risk populations who reside near hazardous and Superfund sites

4.2 Introduction

Cardiovascular disease is the leading cause of death in the developed world and can be modified depending on nutritional habits of the individual. Certain dietary choices have been shown to be pro-atherosclerotic and proinflammatory whereas other, more healthful choices, have been shown to decrease oxidative stress, inflammation, and vascular diseases. An increasing body of scientific research now implicates dietary fats as important mediators of cardiovascular disease. Certain fatty acids such as linoleic acid (parent omega-6 polyunsaturated fatty acid) have been shown to promote endothelial cell inflammation primarily by an increase in toxic linoleic acid metabolites.

Many inexpensive plant derived oils are rich in linoleic acid, which makes this fatty acid a predominate component in Western diets. Reducing the intake of this nutrient has been shown to be protective against multiple proinflammatory diseases[203]. Linoleic acid can be metabolitzed by multiple cell types, including endothelial cells, into bioactive mediators such as cis-epoxyoctadecenoic acids (9, 10- and 12, 13-EOA), 9- and 13- hydroxy-octadecadienoic acid (9- and 13-HODE), and 9- and 13-oxo-octadecadienoic acid (9- and 13-oxoODE)[8, 204]. The formation of these toxic metabolites can be due to random radical-mediated oxidation, or enzymatically controlled by 12/15-lipoxygenases, cyclooxygenases, or even cytochrome P450s[8]. The monoepoxides of linoleic acid are commonly referred to as leukotoxins (LTX) and their abundance is related to cytochrome P450 activation[205]. There is evidence that links these toxic metabolites to organ failure, pulmonary edema, and endothelial cell damage[205]. LTX have been shown to increase endothelial cell permeability and activation of proinflammatory NFκB[205]. There are however, metabolites of linoleic acid that appear to be anti-inflammatory and may be effective protective mediators of endothelial cell dysfunction.

Recently identified endogenous fatty acid metabolites known as nitro-fatty acids have been implicated as possible protective mediators of inflammation and cardiovascular disease. Unsubstituted fatty acids such as oleic acid (18:1), arachidonic acid (20:4), and linoleic acid (18:2) can be nitrated endogenously by multiple enzymatic and non-enzymatic reactions, resulting in the inhibition of pro-inflammatory mediators, induction of protective heme oxygenase-1, and blood vessel relaxation[146]. Nitro-fatty acids can protect via multiple mechanisms including upregulation of PPARγ and Nrf2 as well as downregulation of pro-inflammatory NFκB[147]. ApoE-/- animals administered a

nitro-fatty acid displayed reduced foam cell formation, decreased lesion formation, and inhibited pro-inflammatory adhesion molecule expression compared to vehicle treated control mice[144d]. Recently, Shin et al., reported that nitro-fatty acids may directly modulate endothelial cell signaling, specifically by altering caveolin-1 and eNOS signaling[206]. Vascular endothelial cells are dynamic modulators of CVD and come in contact with heterogenous blood components and stimuli such as a wide assortment of dietary nutrients, lipid soluble toxic pollutants, and endogenous pro-inflammatory signals and compounds. Once believed to be a relatively unimportant regulatory cell type, endothelial cells are now known to be critical mediators of platelet adherence, modulators of vascular tone, regulators of thrombosis, and controllers of immune and inflammatory responses [5]. Since endothelial cells are constantly exposed to blood and associated proteins, it is not surprising that nutrient and chemical compounds from the diet may play a critical role in the regulation of endothelial cell dysfunction and subsequent cardiovascular disease. Due to commonalities between certain dietary nutrients and toxicants in regards to activation of endothelial cells, more research describing the interplay between nutrition and toxicology is critical to better understand the risks of environmental pollutants in a world of multiple concomitant stressors.

Accompanying nutrients from the diet, many lipophilic persistent organic pollutants (POPs) are also distributed throughout the body via blood flow and plasma proteins. Developing a body burden of POPs is not rare; for example, in studies attempting to identify the concentrations of multiple pollutant compounds in human plasma, it is not uncommon for the researchers to find known toxicants in 99-100% of their test subjects in the parts per billion range (ppb)[10]. Not surprisingly, many of these manmade compounds have been shown to interact and activate endothelial cells; some causing toxicity via many of the same mechanisms as detrimental nutrient components. For example, certain toxicants such as PCBs have been shown to increase oxidative stress, upregulate NFκB, and lead to endothelial cell dysfunction and inflammation[12]. Interestingly, it appears that certain nutritional choices, such as diets high in linoleic acid, may actually exacerbate the toxicity of persistent organic pollutants[13].

Previous work has shown that linoleic acid can amplify the endothelial cell toxicity of polychlorinated biphenyls[109]. Linoleic acid pretreatment exacerbated PCB-induced oxidative stress, NFκB DNA binding, and albumin transfer in primary vascular endothelial cells, which mechanistically may be explained by an observed exacerbated

cytochrome P450 1A1 (Cyp1A1) upregulation[109]. Other groups have also documented detrimental nutrient/toxicant interactions; elevated levels of linoleic acid may enhance the cellular bioavailability of PCBs, PCBs can downregulate delta 5 and delta 6 desaturases which are critical for the breakdown of linoleic acid, and PCBs can promote the transport of linoleic acid from circulating plasma into vascular tissues[112, 126]. Finally, it was shown that the exacerbated effects observed may be due to the creation of metabolites such as LTX and LTXD[205]. Although it has been shown that detrimental metabolites of linoleic acid may exacerbate toxicant-induced endothelial cell dysfunction, it is not known if other endogenously formed metabolites such as nitro-linoleic acid may be protective.

Thus, the current study has been designed to investigate mechanistically if pro-inflammatory lipids could increase the toxicity of the coplanar PCB 126, and if nitration of these pro-inflammatory lipids could ameliorate this exacerbated effect. Our data provide evidence that free linoleic fatty acid is pro-inflammatory to vascular endothelial cells, and this toxicity can be exacerbated in the presence of PCBs. On the contrary, our data also provide evidence that nitration of linoleic acid blunts these exacerbated effects. Since linoleic acid makes up a large portion of Western diets, it may be beneficial for future research to investigate mechanisms of endogenously increasing the formation of nitro-fatty acids in humans.

4.3 Materials and methods

4.3.1 Materials and Chemicals

3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) was obtained from AccuStandard Inc. (New Haven, CT). Linoleic and nitro-linoleic acids were purchased from Cayman Chemical (Ann Arbor, MI).

4.3.2 Cell culture and experimental media

Primary vascular endothelial cells were isolated from porcine pulmonary arteries (Han et al., 2010). Cells were cultured in M199 (Gibco, Grand Island, NY), supplemented with fetal bovine serum (FBS; Gibco). Endothelial cells were grown to confluence, followed by incubation overnight in medium containing 1% FBS prior to cell treatment. Stock solutions of linoleic acid or nitro-linoleic acid were purchased or diluted to 1 mg/mL (EtOH) and subsequently diluted to working stocks. Cells were pretreated with fatty acid or vehicle control with total volume being 0.1%. Stock solutions of PCB 126 were prepared in DMSO at 4 mM and subsequently diluted to working stocks in DMSO; control cultures were treated with DMSO vehicle. The levels of DMSO in experimental media were 0.05%. Porcine vascular endothelial cells were treated with PCB 126 at 0.03 nM for 6 h.

4.3.3 Real-time PCR

The levels of mRNA expression were assessed by real-time PCR using a CFX96 Touch Real-Time PCR detection system (BioRad, Hercules, CA) and SYBR Green master mix (Applied Biosystems) as described earlier (Han et al., 2010). Sequences were designed using the Primer Express Software 3.0 for real-time PCR (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences for porcine VCAM-1 and β-actin were described in earlier articles published from our laboratory (Ramadass et al., 2003; Majkova et al., 2009). Porcine NQO1 sequences were: sense, 5′-CCCGGGAACTTTCAGTATCCT-3′; and antisense, 5′-CTGCGGCTTCCACCTTCTT-3′; porcine HO-1 sequences were: sense, 5’-GCCATGTGAATGCAACCCTGTGAA-3’; and antisense, 5’-TTGGGAAAGATGCCACAGACTCCT-3’. Porcine AhR sequences were: sense, 5’-AGCTGCACTGGGCGTTAAA-3’; and antisense, 5’-GCCACTCGCTTCATCAATTCT-3’.

Porcine Cyp1A1 sequences were: sense, 5'-CATCCGGGACATCACAGACA-3'; and antisense, 5'-GCATTCTCGTCCATCCTCTTG-3'. Porcine MCP-1 sequences were: sense, 5'-CGGCTGATGAGCTACAGAAGAGT-3'; and antisense, 5'-GCTTGGGTTCTGCACAGATCT-3'.

4.3.4 Statistical analysis

Data were analyzed using SigmaStat software (Systat Software, Point Richmond, CA). Comparisons between treatments were made by one-way ANOVA or two-way ANOVA as appropriate with post-hoc comparisons of the means (Tukey). To elucidate trends within groups, multiple comparison procedures were completed for tests that displayed overall significance ($p \leq 0.05$-0.1). Interactions between fatty acid treatment and toxicant exposure were denoted. Groups were considered significantly different with a determined p value of $p \leq 0.05$.

4.4 Results

4.4.1 Parent linoleic acid is pro-inflammatory whereas nitro-linoleic acid is anti-inflammatory

Previously, we have shown that linoleic acid can promote endothelial cell dysfunction, as evidenced by a decrease in endothelial barrier function and an increase in LDL transcytosis[109, 207]. These studies used fatty acid concentrations up to 90 μM bound to 30 μM albumen[109]. Here, we treated primary porcine vascular endothelial cells with a low concentration (1-3 μM) of free linoleic fatty acid dissolved in EtOH for 6 h and saw a statistically significant upregulation of VCAM-1 and MCP-1 mRNA expression in a concentration dependent manner (Fig. 4.1, Fig. 4.2). However, this upregulation was not observed in cells treated with nitro-linoleic acid. Interestingly, treatment with nitro-linoleic acid decreased levels of VCAM-1 mRNA approximately 50% compared with ethanol control (Fig. 4.2).

4.4.2 Parent linoleic acid increases AhR and Cyp1A1 expression, but nitro-linoleic acid has no effect

Since it is known that coplanar PCBs exert their toxicity primarily through the AhR, we were interested in determining if linoleic or nitro-linoleic acid would modulate this pathway. Therefore, we treated primary porcine vascular endothelial cells with 1 or 3 μM parent or nitro-metabolite of linoleic acid for 6 hours and harvested for mRNA. Nitro-linoleic acid had no effect on AhR or Cyp1A1 mRNA expression, however, parent linoleic acid significantly increased AhR and Cyp1A1 mRNA expression in a dose dependent manner (Fig. 4.3 and Fig. 4.4).

4.4.3 Nitro-linoleic acid upregulates the antioxidant defense greater than parent linoleic acid

Recently, it has been shown that certain PUFA metabolites may be protective by upregulating the endogenous antioxidant defense. Therefore we treated our endothelial cell model with parent or nitro-linoleic acid for 6 hours and determined mRNA expression levels of the antioxidant enzymes NQO1 and heme oxygenase-1 (HO-1) via real-time PCR. Treatment with parent and nitro-linoleic acid both caused an upregulation of NQO1 at approximately a 4 and 8 fold increased level respectively (Fig. 4.5). Interestingly, HO-1 was only induced in nitro-fatty acid treated cells and this upregulation was considerably higher than observed with NQO1 (Fig. 4.5).

4.4.4 PCBs exacerbate linoleic acid-induced increases in Cyp1A1, but pretreatment with nitro-linoleic acid prevents this exacerbated effect.

In an attempt to determine interactions between diet and toxicant exposure, primary vascular endothelial cells were pretreated with a low level of linoleic or nitro-linoleic acid (0.1 μM) for 6 h and then exposed to physiological relevant levels of PCB 126 (0.03 nM) for another 6 h. Since AhR activation has been shown to be necessary for coplanar PCB toxicity, mRNA levels of the downstream AhR target, Cyp1A1, were investigated via real time-PCR. Cyp1A1 induction has long been used as a surrogate marker for coplanar PCB exposure, and in this study, Cyp1A1 was significantly upregulated in all PCB treatment groups (Fig. 4.6). As was also seen in the experiments described within section 4.4.2 which utilized 1-3 μM of fatty acid, parent linoleic acid significantly increased Cyp1A1 mRNA levels in vehicle control (DMSO) treated cells. Interestingly, in cells that were pretreated with linoleic acid and subsequently exposed to

low concentrations of PCB 126, this increase in Cyp1A1 was significantly exacerbated. However, pretreatment with nitro-linoleic acid returned PCB-induced Cyp1A1 levels back to vehicle control levels and the observed exacerbated effect was lost.

4.4.5 PCBs exacerbate linoleic acid-induced increases in pro-inflammatory mediators, but pretreatment with nitro-linoleic acid prevents this exacerbated effect.

Levels of pro-inflammatory mediators such as MCP-1, VCAM-1, and caveolin-1 were also examined in cells pretreated with fatty acids and subsequently exposed to PCBs. In ethanol control cells, treatment with low dose of PCB for 6 h did not significantly upregulate the pro-inflammatory mediators (Fig. 4.7). Parent linoleic acid significantly increased MCP-1 and VCAM-1 mRNA levels in DMSO treated cells. Interestingly, in cells that were pretreated with linoleic acid and subsequently exposed to low concentrations of PCB 126, this increase in MCP-1 and VCAM-1 was significantly exacerbated. However, pretreatment with nitro-linoleic acid returned PCB-induced MCP-1 and VCAM-1 levels back to control vehicle levels and the observed exacerbated effect was lost.

4.5 Discussion

Emerging data now implicate both positive and negative effects related to the interplay between nutrition and toxicology. Originally, food from the diet were believed to be an exposure route of toxicants. However, evidence suggests that a person's diet, and perhaps more appropriately the specific nutrients found within one's diet, may either exacerbate or prevent toxicant-induced diseases[13]. The protective or detrimental interactive effects observed may be due to commonalities between toxicant and nutrient signaling pathways. Nutrients and toxicants can both modulate transcription factors and signaling mediators related to redox balance, stress response, and inflammation. Endothelial cells are prime candidates to use as a tool to examine the interplay between toxicology and nutrition because this cell type normally comes into contact with an array of circulating nutrients and pollutants. Dysfunction of endothelial cells can occur by toxicants and be exacerbated by certain pro-oxidative and/or pro-inflammatory diet derived lipids, which can lead to atherosclerosis and vascular related diseases.

Atherosclerosis is a disease of lifelong progression and can be modulated by many controllable and non-controllable risk factors. Dietary habits, sedentary lifestyle, and smoking may all be risk factors related to choice, but pollutant exposures leading to heart disease risk may occur invisibly. Low level exposures occur daily to a wide variety of environmental stressors including chlorinated toxicants, endocrine disrupting chemicals, and heavy metals, which by themselves may not elicit life threatening reactions, but in combination as daily pollutant mixtures or in conjunction with other poor lifestyle choices (e.g., poor nutritional choices or sedentary lifestyle) may increase the risk for multiple pro-inflammatory chronic diseases. The results described within this chapter provide evidence that certain dietary nutrients can exacerbate the toxicity of physiologically relevant low levels of persistent organic pollutants. Also, it was determined that the addition of an NO_2 moiety to form the nitro-fatty acid version of linoleic acid effectively eliminated this exacerbated toxicity. This observation is of importance because linoleic acid can be endogenously nitrated within the body, but the control mechanisms regulating nitro-fatty acid formation are not well understood.

Nitro-fatty acids are a group of endogenously formed bioactive lipid metabolites that have been examined for protective roles in multiple chronic diseases. Nitro-oleic and nitro-linoleic acid are two highly studied fatty acid derivatives due to their observable presence in human plasma and anti-inflammatory nature in cell and mouse models of disease. Nitro-oleic acid has been shown to prevent against angiotensin II- induced hypertension, endotoxin-induced endotoxemia, and LPS-induced lung injury[208]. Researchers may be focused primarily on nitro-oleic acid in an attempt to identify novel mechanisms of protection from the consumption of oleic acid-rich extra virgin olive oil. In this current study, treatment with nitro-linoleic acid increased the expression of protective HO-1 (a Nrf2 controllable antioxidant enzyme) and decreased pro-inflammatory mediators, especially when compared to cells treated with pro-inflammatory parent linoleic acid. Interestingly, both nitro-linoleic and parent linoleic acid increased the expression of GST. GST has binding sites for both Nrf2 and AhR, and in this current study it was observed that parent linoleic acid treatment upregulated AhR[209]. AhR and Nrf2 can work in concert to eliminate compounds from the body, but sometimes AhR activation can lead to the creation of even more toxic metabolites. Identifying the causative bioactive nutrients within an overall healthful diet is an emerging but still difficult field of study. Arguably, it may be even more helpful to identify detrimental

nutrients and nutrient metabolites that can detrimentally effect health outcomes or act as biomarkers.

The endogenous metabolism and breakdown of nutrients can lead to the formation of mediators linked to inflammation and chronic disease. Specifically related to this chapter, the parent omega-6 fatty acid Linoleic acid can be oxidized enzymatically and non-enzymatically to produce possibly harmful byproducts. Interestingly, cytochrome P450s have been shown to be critical mediators of both PCB toxicity and the formation of toxic linoleic acid metabolites called leukotoxins (LTX and LTXD). In this current work, it was determined that treatment for 6 h with linoleic acid upregulated Cyp1A1 mRNA levels, which has routinely been utilized as a marker for AhR activation. The observed upregulation of Cyp1A1 may have been due to the observed AhR message increase, but others have shown that Cyp1A1 can be upregulated by fatty acids and fatty acid metabolites in a PPARα-mediated mechanism[210]. In similar previous work, 6 h treatment with linoleic acid lead to an upregulation of another CYP family member Cyp2C9 and ultimately leading to superoxide formation and oxidative stress[211]. Our current work did not examine oxidative stress conditions in our porcine endothelial cell model, but did look at downstream markers of inflammation such as MCP-1 and VCAM-1. In each of these cases linoleic acid was pro-inflammatory. Certain omega-6 fatty acids and their metabolites are known to be toxic to endothelial cells. It is unknown what levels of toxic linoleic acid metabolites are formed within the cell after the exposure conditions in this current work, but others have reported toxicity of linoleic acid metabolites such as LTX and LTXD at concentrations of 40 μM[205]. These low levels were shown to increase albumin transfer and cause endothelial cell dysfunction. Linoleic acid is found abundantly in Western diets and may reach 8-12 grams per day in adults[210]. Lipid peroxidation products of linoleic acid also exist, and in fact, the pool of peroxidation productions is dominated by linoleic acid-based compounds[212]. Cellular lipoxygenases generate lipid hydroperoxides and reactive oxygen species and the major metabolites formed from linoleic acid are 9-hydroperoxy-10,12-octadienoic acid (9-HPODE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE). Lipid peroxidation can also lead to the formation of highly reactive and toxic metabolites such as 4-hydroxynonenal (4-HNE)[212]. Levels of these toxic metabolites were not examined in this current work, but would be a beneficial mechanistic link between the observed toxicity of parent but not nitro-linoleic acid. Perhaps the addition of the NO_2 to the linoleic acid can prevent or slow the enzymatic and non-enzymatic production of toxic

metabolites. Therefore, it may be critical to better understand the formation of toxic and protective linoleic metabolites to better identify and communicate risks with eating diets high in omega-6 fatty acids.

Understanding the interplay and interactions between toxicants and nutrients will allow for more effective exposure risk assessments. The exacerbated effect of certain nutrients on toxicological outcomes has been proposed previously and has been seen with multiple toxicants and nutrient combinations[13]. In this work, pretreatment with linoleic acid but not nitro-linoleic acid exacerbated PCB-induced Cyp1A1, VCAM-1, and MCP-1 upregulation. This effect may be linked to PCB 126 and linoleic acid treatments both being able to activate the AhR and subsequently increase oxidative stress conditions. It would be interesting to examine the interactive effects between a nutrient and toxicant that activate dissimilar pathways. Pretreatment with nitro-linoleic acid may be protective by not sensitizing AhR prior to toxicant exposure and in addition may activate Nrf2 and the antioxidant response. As seen in Chapter 3 of this dissertation, dietary interventions upregulated the antioxidant response which allowed for a more efficient and effective physiological response in the presence of PCB.

In summary, our current study supports our previous work illustrating that a major dietary nutrient found in Western diets, linoleic acid, is pro-inflammatory to endothelial cells and exerts exacerbated effects in the presence of PCBs. Interestingly, our current *in vitro* data contribute to the overall hypothesis that certain nutrients can decrease the toxicity of environmental insults. Although the parent linoleic acid appeared to be pro-inflammatory, pretreatment with the endogenously created metabolite, nitro linoleic acid, prevented this exacerbated effect. More studies are needed to further understand detailed mechanisms of the creation of endogenously controlled nutrient metabolites and how to possibly shift the pool of bioactive lipids from detrimental to positive.

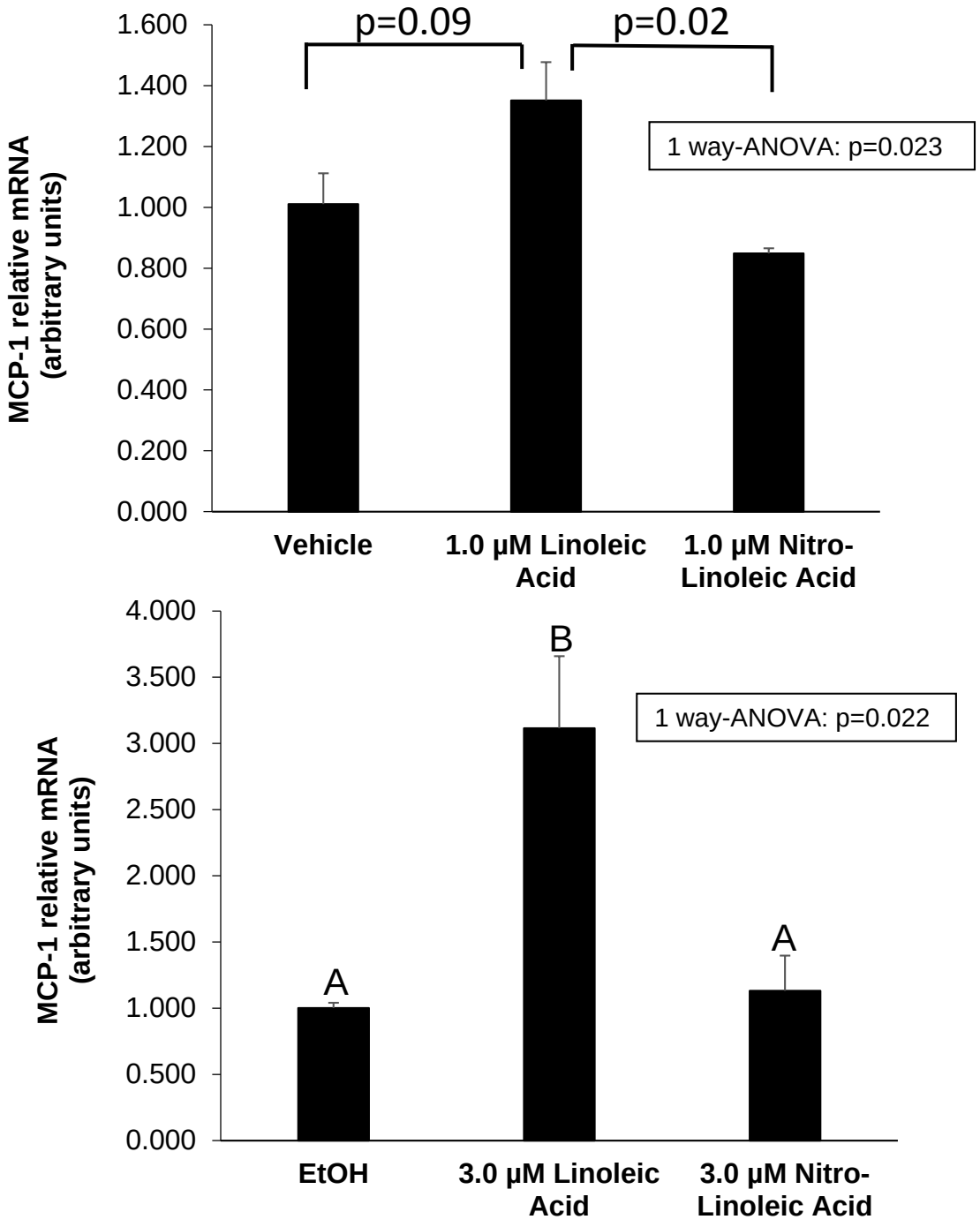

Fig. 4.1 Relative mRNA levels of monocyte chemoattractant protein-1 in cells treated for 6 h with parent or nitrated linoleic acid (1 or 3 μM). Parent linoleic acid was pro-inflammatory and increased MCP-1 levels but this increase was not seen in nitro-fatty acid treated cells. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). Different letters denote statistical significance between treatment groups.

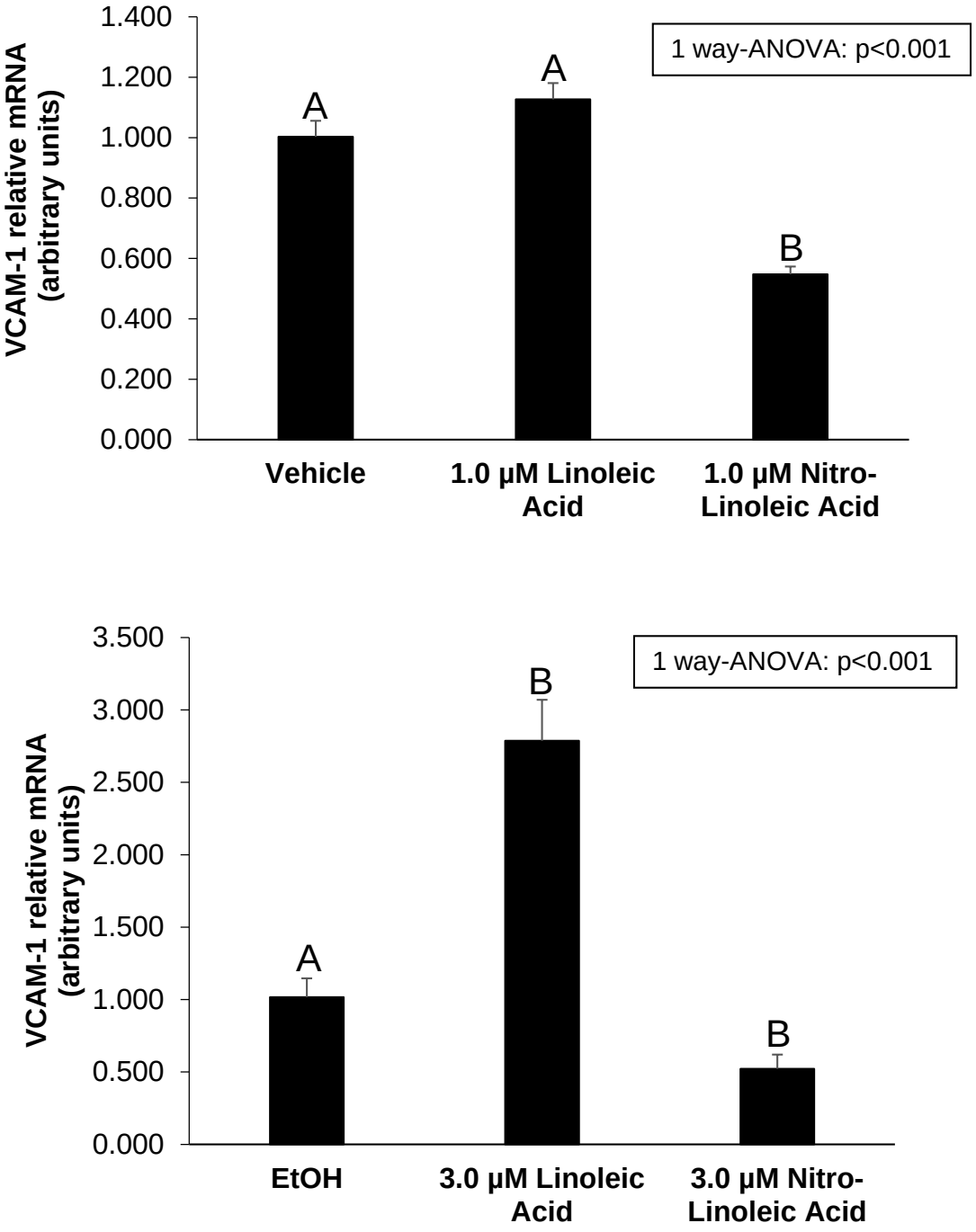

Fig. 4.2 Relative mRNA levels of vascular cell adhesion molecule-1 in cells treated for 6 h with parent or nitrated linoleic acid (1 or 3 μM). Parent linoleic acid was pro-inflammatory and increased VCAM-1 levels but nitro-fatty acid treatment was anti-inflammatory. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). Different letters denote statistical significance between treatment groups.

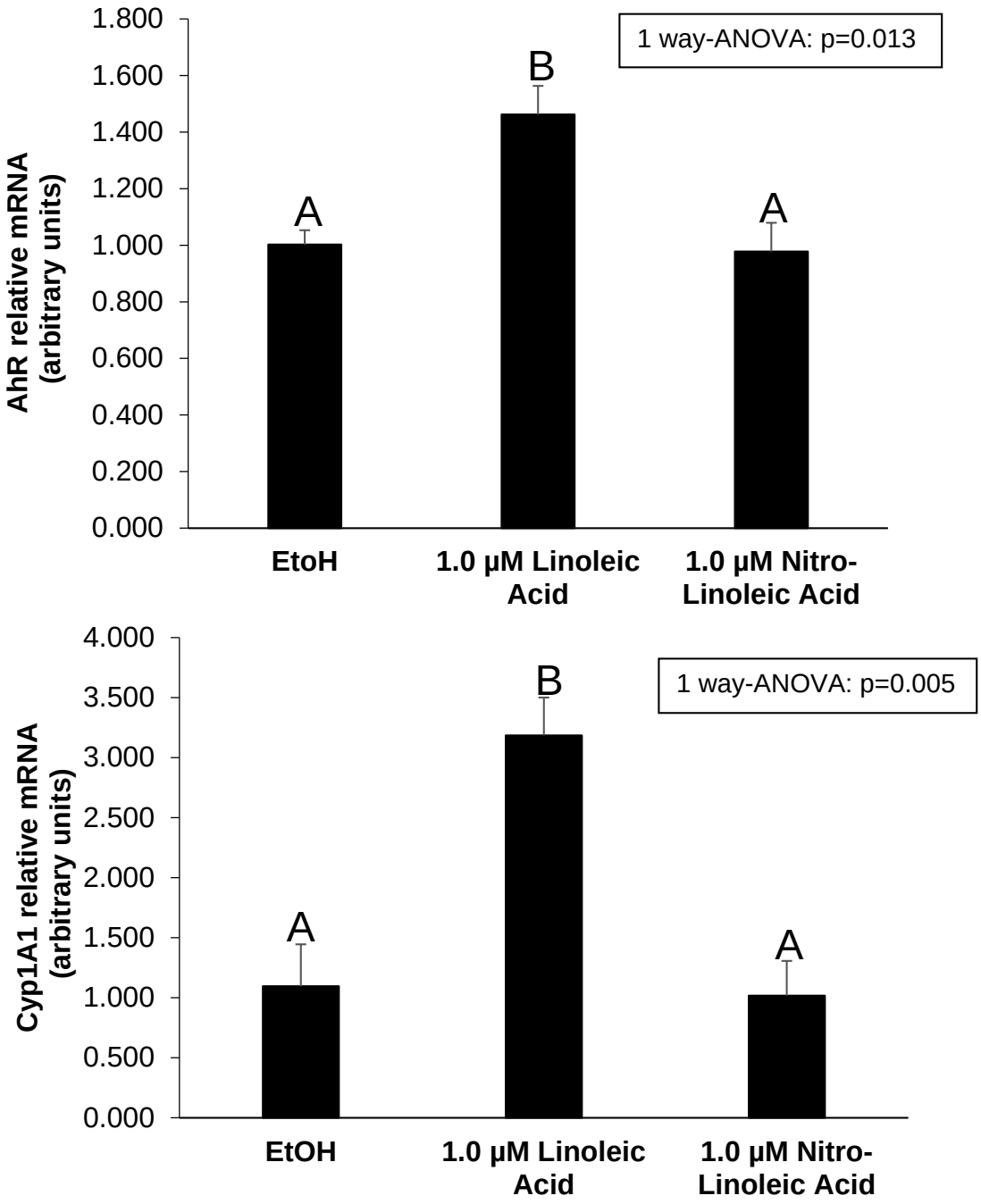

Fig. 4.3 Relative mRNA levels of Aryl hydrocarbon receptor and cytochrome P4501A1 in cells treated for 6 h with parent or nitrated linoleic acid (1 µM). Parent linoleic acid upregulated AhR as well as Cyp1A1, but this upregulation was not seen in nitro-fatty acid treated cells. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). Different letters denote statistical significance between treatment groups.

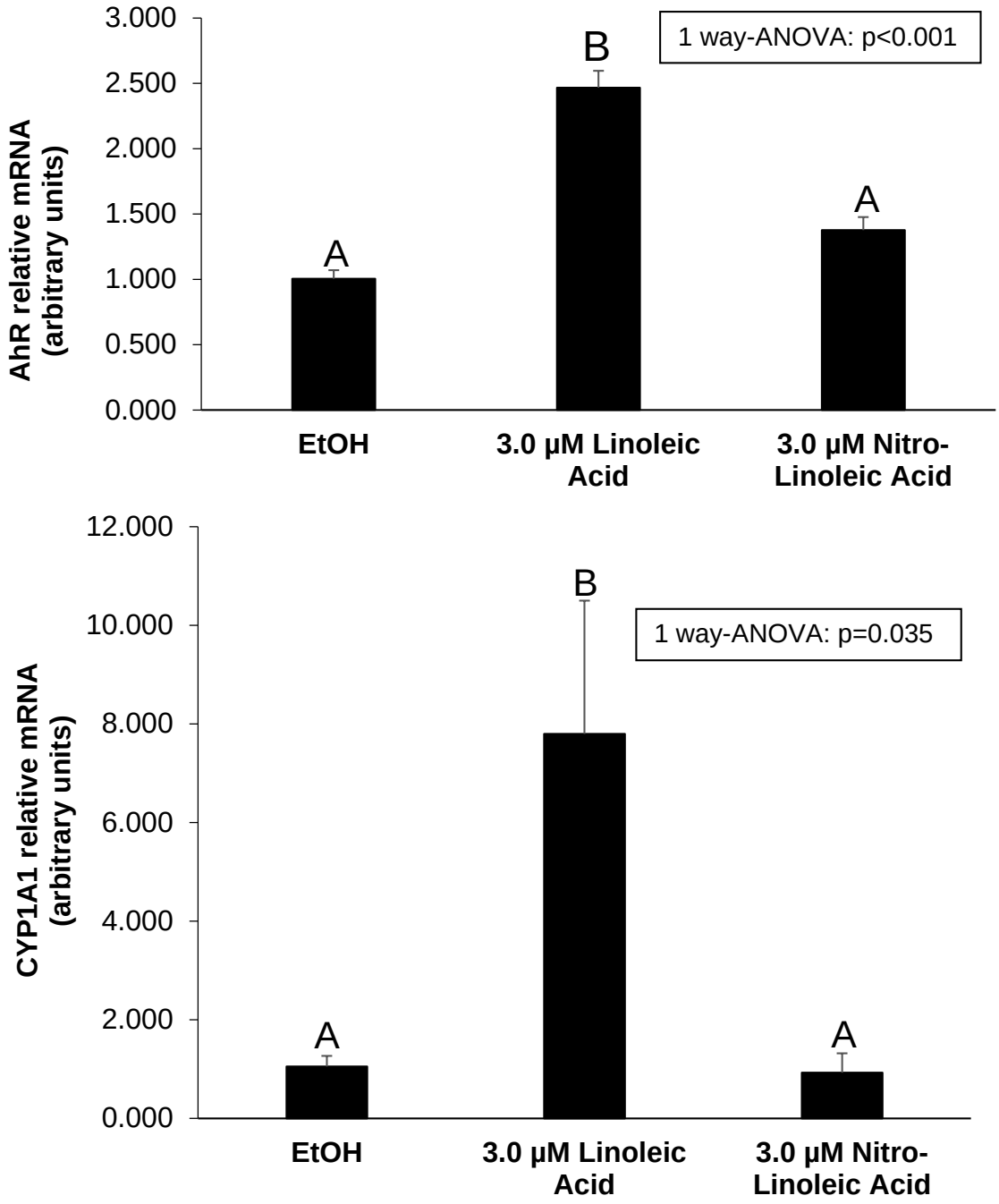

Fig. 4.4 Relative mRNA levels of aryl hydrocarbon receptor and cytochrome P4501A1 in cells treated for 6 h with parent or nitrated linoleic acid (3 µM). Parent linoleic acid upregulated AhR as well as Cyp1A1, but this upregulation was not seen in nitro-fatty acid treated cells (ptrend=0.09). All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). Different letters denote statistical significance between treatment groups.

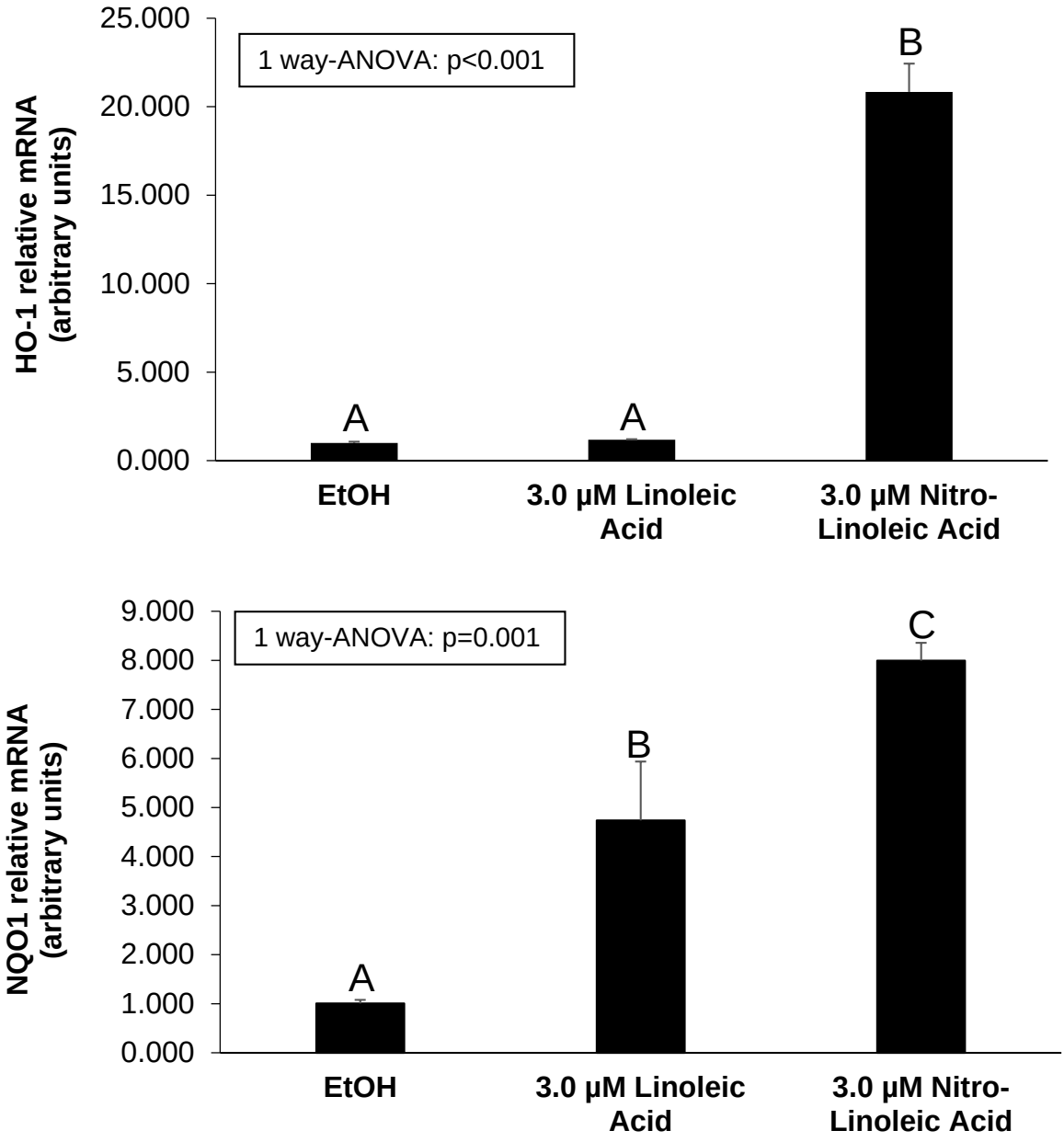

Fig. 4.5 Relative mRNA levels of heme oxygenase-1 and NADPH quinone oxido-reducatase-1 (antioxidant enzymes) in cells treated for 6 h with parent or nitrated linoleic acid (3 µM). Parent linoleic acid upregulated NQO1, but not HO-1 whereas nitro-fatty acid upregulated both protective enzymes. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). Different letters denote statistical significance between treatment groups.

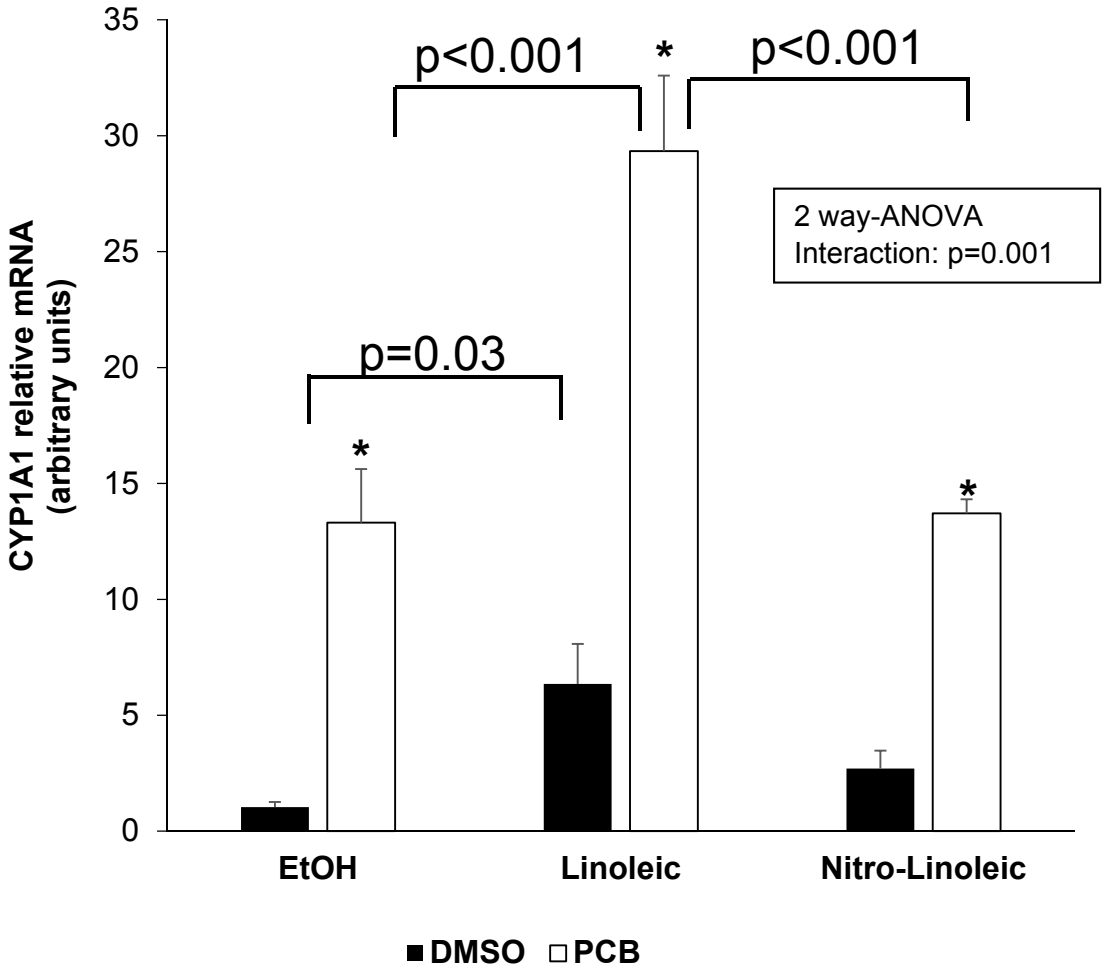

Fig. 4.6 Relative mRNA levels of cytochrome p4501A1 in cells pretreated for 6 h with parent or nitrated linoleic acid (0.1 μM) and subsequently treated for 6 h with 0.03 nM PCB 126. Cells treated with PCB 126 exhibited an upregulated CYP1A1 response (*p<0.05)**.** This upregulation of Cyp1A1 was exacerbated in cells treated with parent linoleic acid, but not in cells pretreated with nitro-fatty acid. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). * denotes p<0.05 compared to corresponding DMSO control.

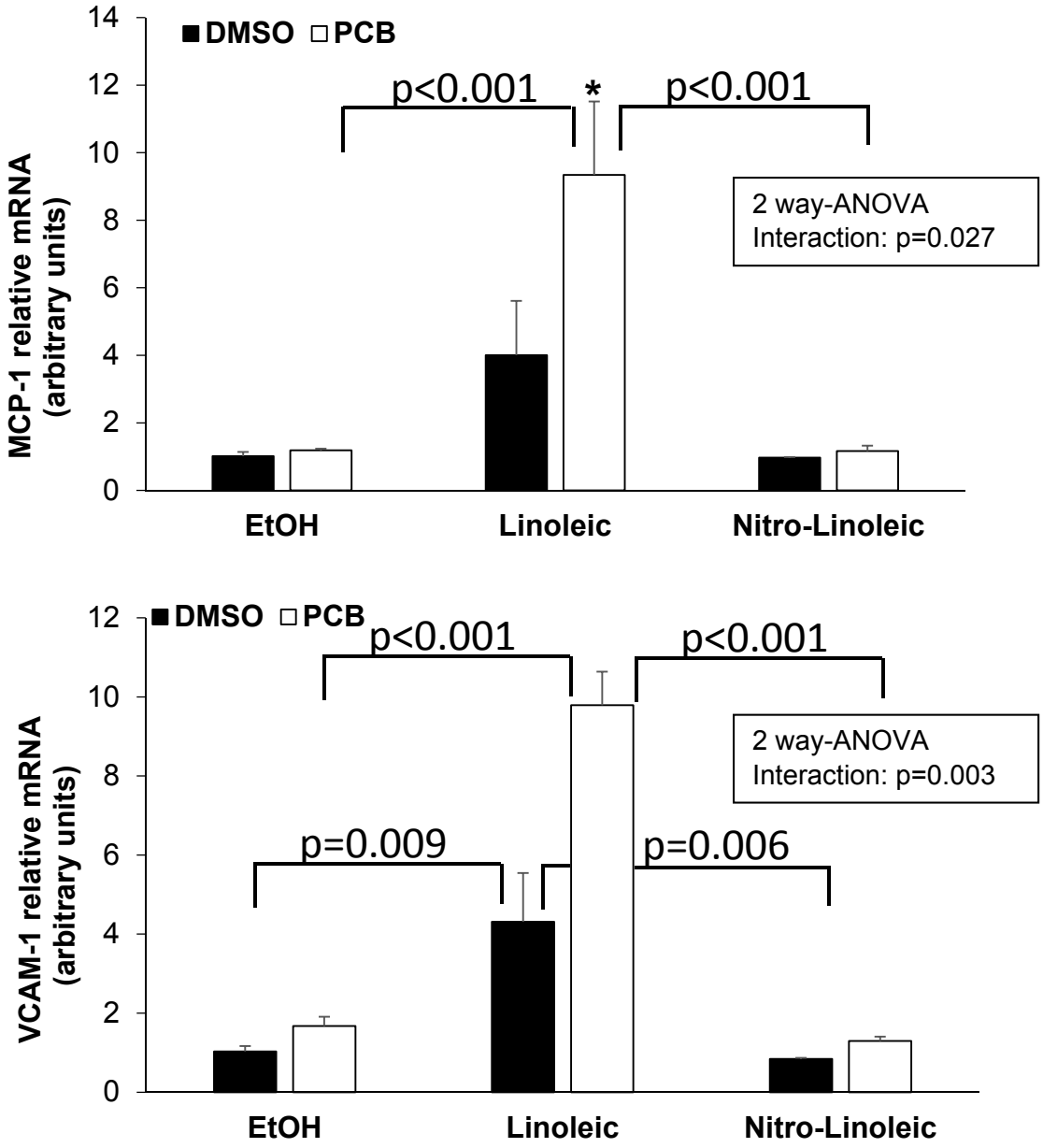

Fig. 4.7 Relative mRNA levels of MCP1 or VCAM-1 in cells pretreated for 6 h with parent or nitrated linoleic acid (0.1 μM) and subsequently treated for 6h with 0.03 nM PCB 126. Control cells treated with PCB 126 exhibited low induction of the pro-inflammatory markers after an acute exposure to PCB (6 h). Parent linoleic acid was pro-inflammatory and significantly exacerbated the PCB effect. Cells pretreated with nitro-fatty acid mirrored control cells' responses. All values were determined using the relative quantification method (ΔΔCt) as compared to EtOH control. Data are presented as mean±S.E.M (n=3). * denotes $p<0.05$ compared to corresponding DMSO control.

Chapter Five. Overall discussion

5.1. Discussion

5.1.1 Summary

The research presented within this dissertation provides new mechanistic toxicological insights into polychlorinated biphenyl-induced endothelial cell dysfunction and inflammation. Ultimately, we are interested in decreasing the negative health risks associated with pollutant exposures; thus this dissertation also provides novel evidence that diets high in healthful bioactive nutrients may be a sensible means of modulating associated disease risks. Caveolae harbor numerous inflammatory proteins, and Nrf2 is considered the master antioxidant controller in most cell types including those that make up vascular tissues. Caveolae and Nrf2 pathways are critical mediators of PCB-induced endothelial cell dysfunction as well as nutritional modulation of PCB vascular toxicity. Decreasing Cav-1 in endothelial cells prevents PCB-induced upregulation of pro-inflammatory mediators, and this protection may be due to a direct upregulation of protective antioxidant enzymes under the control of the transcription factor Nrf2. Dietary intervention strategies utilizing flavonoid or bioactive fatty acids can modulate the toxicity of coplanar PCB 126. We demonstrated that a diet enriched in green tea polyphenols can decrease oxidative stress and inflammation in mice subsequently exposed to PCB. Finally, we also examined the role of bioactive nutrient metabolites, such as the endogenously formed nitro-fatty acids, as possible novel modulators of toxicant-induced endothelial cell dysfunction.

5.1.2 Decreasing Cav-1 upregulates the Nrf2 antioxidant response

Endothelial cells come in to contact with an array of persistent organic pollutants daily and have evolved multiple means of protecting against associated cellular stresses. Coplanar PCBs, the most toxic to endothelial cells, have been shown to induce an increase in ROS and RNS primarily through an AhR and Cyp1A1 mediated mechanism[12b, 12c]. AhR has been shown to locate and concentrate within caveolae lipid domains and specifically has been shown to co-immunoprecipitate with the major structural protein of caveolae, Cav-1[12b]. In fact, treatment of endothelial cells with coplanar PCBs induces Cav-1 expression and increases this partnership with AhR. Our

laboratory determined that loss of Cav-1, either through siRNA knockdown in cells or the use of Cav-1 null mice, was protective and mitigated PCB-induced endothelial cell dysfunction and the earliest stages of PCB-induced atherosclerosis[12a, 23b]. Multiple hypotheses were postulated to explain this protection including decreased uptake of PCBs into endothelial cells, modulation of AhR signaling due to the loss of Cav-1, and perhaps modulation of cross-talk with other signaling partners related to inflammation and PCB-induced endothelial cell toxicity. This dissertation has investigated this final hypothesis and determined that Cav-1 signaling can modulate the antioxidant defense within a cell by altering the Nrf2 pathway.

Recently it has been shown that downregulation of Cav-1 leads to an increase in protective HO-1, an antioxidant controlled by Nrf2[116b]. This observation led us to investigate mechanistically the link between Cav-1 and Nrf2. We determined that decreasing the protein levels of Cav-1 upregulated Nrf2 activation leading to an increase in cellular levels of multiple antioxidant enzymes and ultimately prevented PCB-induced endothelial cell activation. Interestingly, two other independent research groups also recently published similar results detailing Cav-1's inhibitory role of Nrf2[98, 140, 160]. These highly mechanistic works focused on direct inhibition of Nrf2 through Cav-1's binding domain. Their work suggests that one of the interaction mechanisms linking Cav-1 and Nrf2 pathways is a direct binding and inhibition, much like in the case of eNOS/Cav-1 interaction. The work described within this dissertation details other possible mechanisms of Cav-1 Nrf2 cross-talk, including the modulation of Nrf2 inhibitor proteins such as Keap1 and Fyn kinase. Mechanisms linking the downregulation of Cav-1 levels to also downregulation of Keap1 and Fyn are unknown and present the possibility for multiple future studies. A better understanding of this "blackbox" could lead to new therapeutic targets that ultimately activate Nrf2 and prevent inflammation and oxidative stress.

The crosstalk between caveolae and Nrf2 signaling pathways appears to be evolutionarily conserved among mammalian species. A strength of chapter two of this dissertation is that cross-talk between Cav-1 and Nrf2 was observed in three distinct species; human, pig, and mouse. This may support the hypothesis that caveolae and specifically Cav-1 are critical mediators of inflammation and oxidative stress within endothelial cells, and targeted downregulation of endothelial cell Cav-1 may protect against atherosclerosis, inflammation, and toxicant-induced disease. A goal of our

laboratory is to determine therapeutic bioactive nutrients that can modulate caveolae and/or Nrf2 signaling mechanisms and ultimately protect against environmentally-induced stress and disease.

5.1.3 A diet high in green tea polyphenols modulates the toxicity of PCB 126

Nutrients found within fruits and vegetables are gaining much attention for their possible anti-inflammatory and cardioprotective effects. Our laboratory has focused primarily on flavonoids and polyphenols and their protective effects related to PCB-induced endothelial cell dysfunction. Recently we determined, in our porcine endothelial cell model, that pretreatment with a flavonoid found within green tea, epigallocatechin gallate (EGCG), limited PCB-induced upregualtion of MCP-1 and VCAM-1. This protection may have been due to an increase in Nrf2 controlled antioxidant genes in the cells pretreated with the bioactive nutrient[73b]. In work described within chapter three of this dissertation we attempted to further substantiate this previous study by supplementing mice with a diet high in green tea catechins and subsequently treating with low level does of coplanar PCB.

Green tea supplementation in the diet had interesting effects on PCB toxicity and overall was anti-inflammatory when in the presence of PCB. We originally hypothesized, based off of previous cell culture work, that supplementation with green tea catechins would increase Nrf2 activation, decrease AhR signaling, and ultimately act as an anti-inflammatory agent in both vehicle and PCB-treated groups. Interestingly, we saw barely any green tea-induced upregulation of Nrf2-controlled genes in the associated DMSO group, but saw drastic and consistent responses in mice fed a green tea supplemented diet and subsequently exposed to PCBs. Concerning AhR, green tea supplementation did not decrease basal levels of AhR or AhR targets such as Cyp1A1 and Cyp1B1, but interestingly caused an increase in these three targets in mice supplemented with green tea and subsequently exposed to PCB. Ultimately however, we determined that the unexpected upregulation of both AhR and Nrf2 in the concomitant group resulted in a significant decrease in *in vivo* oxidative stress markers and a downregulation of pro-inflammatory mediators. Our current working hypothesis is that the green tea extract worked as a hormetic and ultimately primed the mouse's antioxidant and xenobiotic defenses prior to the subsequent PCB exposure. Thus, once a secondary stressor was encountered, a set of more efficient and effective anti-

inflammatory events occurred. AhR upregulation can lead to increased metabolism and excretion of xenobiotics and this was examined partially in this study. Unfortunately, feces and urine were not collected and only plasma levels of PCB 126 and PCB126-OH metabolites were examined. We believe that certain nutrients can increase the excretion of toxicants ultimately decreasing the body burden and decreasing the potential toxicity of lipid soluble pollutants. Proof of principle studies have investigated this hypothesis and the exciting results garner further future investigation[129a, 133a]. Although the mice were supplemented with an extract of green polyphenols, more than likely the individual catechins were quickly metabolized to multiple breakdown products. An emerging field of nutrition now is interested in determining the kinetics of formation and pharmacological effects (either positive or negative) of nutrient metabolites. It is unknown if the interaction between PCBs and green tea diet altered the cellular pool of catechins or catechin metabolites, but this would be an interesting future study that may help mechanistically explain protection observed only in the concomitant group. In chapter four of this dissertation, this emerging area of nutrition research was examined by comparing the detrimental and protective effects of parent versus an endogenous metabolite using an omega-6 fatty acid as a model.

5.1.4 Parent linoleic acid exacerbates PCB-induced endothelial cell dysfunction whereas treatment with the endogenously formed metabolite, nitro-linoleic acid, is anti-inflammatory.

The endogenous metabolism of nutrients can lead to toxic and/or protective bioactive mediators. Many inexpensive plant derived oils are rich in linoleic acid, which makes this fatty acid a predominate component in Western diets. Reducing the intake of this nutrient has been shown to be protective against multiple proinflammatory diseases[203]. Linoleic acid can be broken down by multiple metabolic enzymes including cytochrome P450s leading to toxic metabolites such as leukotoxin (LTX) [205]. However, emerging evidence now shows that linoleic acid can be metabolized into protective anti-inflammatory mediators such as the recently identified nitro-linoleic acid. Nitro-fatty acids can activate Nrf2 and inhibit pro-inflammatory NFκB, and in mouse models of disease, have been shown to prevent atherosclerosis, sepsis, and vascular injury[144c, 144d, 147a, 208a]. Understanding the cellular control mechanisms governing the formation of either protective or detrimental metabolites is important, but more work is needed so that

these pathways can be effectively manipulated. Western diets are high in linoleic acid but can also include other pro-inflammatory compounds that result in exacerbated toxic effects.

Environmental pollutants such as lipophilic polychlorinated biphenyls enter the body primarily through ingestion and can be stored for long durations in adipose tissues. Endothelial cells are prime candidates to study the interactions between pollutants and nutrients because this cell type routinely comes in to contact with a myriad of compounds via the circulation. Our laboratory previously reported that endothelial cells pretreated with an omega-6 fatty acid were at greater risk for PCB-induced complications including exacerbated oxidative stress and decreased endothelial cell barrier function[109]. Also, it was determined in a more recent study that pretreatment with linoleic acid exacerbated tumor necrosis factor alpha (TNF-alpha) induced oxidative stress and endothelial cell dysfunction[213]. Interestingly, caveolae and Cav-1 may be upregulated both by linoleic acid and coplanar PCBs, and silencing of Cav-1 has been shown in our laboratory to prevent linoleic acid as well as PCB-induced endothelial cell activation[23b, 214]. A recent publication implicated nitro-fatty acids as possible downregulators of Cav-1, so we investigated the hypothesis that pretreatment with nitro-linoleic acid could prevent PCB-induced endothelial cell toxicity. In our current study we again determined that linoleic acid was pro-inflammatory, when treated alone, as evidenced by increases in expression levels of MCP-1 and VCAM-1. The levels of these pro-inflammatory mediators were significantly exacerbated once PCB added. On the contrary, treatment with nitro-linoleic acid was anti-inflammatory and did not result in an exacerbated PCB response. Importantly, the low concentrations of PCB utilized within this study mirror blood levels found in humans, and since linoleic acid is found also at high levels in humans, interactive exacerbatory effects may be physiologically common.

5.2 Future directions and conclusions

The interplay between nutrition and toxicology is a growing field of discovery worthy of intense investigation. Data described within this dissertation and elsewhere now implicate either a protective or detrimental modulatory role of nutrients on the toxicity of environmentally relevant pollutants. Numerous synthetic chemicals exist that may exert stress on physiological systems, and countless more emerging pollutants will be introduced into the environment as a growing number of countries become ever more

industrialized. Toxicologists have long studied the detrimental effects of individual compounds, and their perseverance has helped to impact change at the highest levels of industry and government. However, as body burdens of complex mixtures increase and the production of potential dangerous compounds overtakes the capacity for in-depth toxicity testing, it is important to identify sensible means of biomedically reducing the risks of environmental pollutant-induced diseases.

There is strong evidence that diets rich in bioactive lipids and plant-derived polyphenols protect against cardiovascular diseases generally[215]. This may be due in part to a significant improvement in endothelial function and a decrease in inflammatory cytokines[216]. Polyphenols (e.g., flavonoids and other bioactive compounds) are found in fruits and vegetables[217] and share antioxidant and anti-inflammatory properties[218]. The health effects of polyphenols depend on the amount consumed and on their bioavailability[217, 219]. Little is known about the effects of bioactive lipids and other antioxidant and anti-inflammatory plant-derived nutrients (e.g., polyphenols) on environmental contaminant-induced cytotoxicity. Potential protective mechanisms of nutrients may involve modification of AhR and CYP1A1 expression and activation[200, 220]. Our own data suggest that protective effects of bioactive fatty acids and flavonoids are initiated upstream from Cyp1A1 and that these nutrients may be of value for inhibiting the toxic effects of PCBs on vascular endothelial cells [97, 221].

We hypothesize that bioactive compounds common in fruits and vegetables protect the vascular endothelium against toxic insults by induction of Nrf2 signaling and subsequent upregulation of detoxifying or antioxidant enzymes. We have generated evidence indicating that the complex interplay between toxicant exposure and nutritional profile is integral to the development of new multi-faceted, thus more precise, risk assessment models that accomplish a more comprehensive set of perspectives on true risk to an at-risk population. Traditional risk assessment is limited to studying the harmful effects to human health/ecological systems resulting solely from exposure to an environmental stressor. However, multiple stressors, chemical and non-chemical in origin, likely contribute to the overall disease risk that is related to exposure to a toxicant or mixture of pollutants. To better qualify cumulative risk in an individual or a population, non-chemical stressors, including psychosocial, physical, and/or dietary components need to be considered. We propose a paradigm shift whereby nutritional status or dietary health is a critical player in the overall vulnerability to environmental

stressors, and thus nutrition must be considered in overall risk assessment[222]. A body of evidence suggests that healthful nutrition offers a viable modulator that could markedly buffer the body against toxic chemical insult from persistent chlorinated organics.

Multiple future directions can be examined related to the work described within this dissertation. First off, our laboratory has long investigated the role endothelial cell caveolae play in PCB-induced endothelial cell dysfunction. Endothelial cell dysfunction is just one important component of the initiation of atherosclerosis, and impacts of immunological cells such as macrophages may also play a critical role. Interestingly, Cav-1 appears to be critical for the differentiation of monocytes into macrophages and Cav-1 is increased once monocytes adhere to the endothelium[223]. Therefore in the future we could examine the impact of downregulating Cav-1 in macrophages and the impact on PCB-induced vascular toxicity. It would be interesting to observe similar Cav-1 Nrf2 crosstalk in macrophages and other vascular related cell types.

Another future direction for the laboratory will be to continue to better identify and mirror true human exposures to environmental pollutants. Throughout this dissertation we have utilized a single PCB congener, PCB 126, as a model toxicant. Since human exposures to a single PCB congener rarely occur, we could study primarily PCB 126 and a simple mixture consiting of a coplanar (126), noncoplanar (153) and mixed congener (118) PCBs, all of which are detectable in human serum at significant levels[224; 46]. Concentrations of the individual congeners to be used in these studies would reflect levels found in human plasma[225]. Our laboratory has long been priviledged to be part of the NIEHS-funded Superfund Research Program, and this funding has allowed for us to create strong collaborations with analytical instrumentation experts that can help identify concentrations of a wide variety of environmental pollutants within plasma and tissues of people. Also, these collaborations have helped our nutritional modulation studies by identifying levels of bioactive mediators within our samples. We plan to identify biomarkers which can predict successful nutritional interventions against environmental pollutants and associated diseases. Personally, I have worked with the Superfund Research Support Core to identify levels of nitro-fatty acids in human and mouse plasma, and hopefully these preliminary studies can lead to future directions for our laboratory (see Appendix A). It would be extremely interesting to identfiy correlations between nutrition, toxicant body burden, and predisposition to cardiovascular disease in populations residing near hazardous waste sites.

In conclusion, we were able to demonstrate that PCB 126 induces endothelial cell dysfunction in a Cav-1 mediated mechanism. Downregulating Cav-1 was shown to upregulate the protective Nrf2 pathway which helped to block PCB-induced endothelial cell toxicity. We have shown that dietary nutrients can modulate the toxicity of PCBs through Cav-1 and/or Nrf2 pathways, and nutritional modulation may be an effective means of preventing toxicant-associated diseases. Within this dissertation, we demonstrated that a flavonoid-rich diet could decrease oxidative stress and inflammation when in the presence of PCB. Additionally, we also showed that certain pro-inflammatory nutrients could exacerbate PCB-induced endothelial cell toxicity whereas a bioactive nutrient metabolite was anti-inflammatory. Together, these data support the paradigm that nutritional modulation may be a sensible means of reducing disease risks associated with exposure to environmental pollutants.

Appendices

Appendix A. Novel analytical methodologies to study anti-inflammatory nitro-fatty acids

A.1 Synopsis

It is widely hypothesized that the possible beneficial health effects of certain polyunsaturated fatty acids involves their enzymatic or non-enzymatic conversion to biologically active metabolites. Accordingly, definitive identification and quantitation of bioactive fatty acid derivatives is essential for both evaluating the beneficial effects of these nutrients and identifying biological markers to monitor the therapeutic efficacy of fatty acid administration in clinical or preclinical settings. Nitro-fatty acids are a class of fatty acid metabolites that are formed by chemical nitration of the double bonds of unsaturated fatty acids, and in mouse models, nitro-fatty acids decrease systemic oxidative stress and inflammation suggesting that these could be biologically relevant mediators. However, in large part due to inherent limitations of currently used analytical approaches for the detection and quantitation of free and esterified nitro-fatty acids, many questions remain to be elucidated regarding their abundance endogenous metabolism and biological actions. In this study we developed a sensitive and specific method for detection and quantitation of nitro fatty acids by combining chemical derivatization with electrospray ionization high resolution tandem mass spectrometry. This method involves formation of methyl pyridinium derivatives of nitro-fatty acids which ionize very strongly in positive mode resulting in increased sensitivity of more than 500-fold over underivatized fatty acids monitored in negative ionization mode. These derivatives were analyzed by HPLC electrospray ionization tandem mass spectrometry using an AB Sciex 5600 quadrupole TOF MS. Together, these methods allow us to identify the ions of the parent nitro-fatty acid derivative at high resolution and confirm their identities by high resolution analysis of their product ion spectra. To validate these methods, we demonstrate detection and quantitation of nitro oleate in plasma following intraperitoneal administration to mice. In conclusion, we have developed and validated novel analytical methodologies to study anti-inflammatory nitro-fatty acids which may

aide in advancing research of nitro-fatty acids as a therapeutic agent against inflammation and atherosclerosis.

A.2 Introduction

Nitro-fatty acids are a relatively newly discovered class of modified lipids that exhibit anti-inflammatory properties via multiple mechanisms. Unsubstituted fatty acids such as oleic acid (18:1) and arachidonic acid (20:4) can be nitrated endogenously by multiple enzymatic and non-enzymatic reactions such as peroxynitrite mediated events[145]. The addition of NO_2 moieties to unsaturated fatty acids has been shown to result in generation of strongly anti-inflammatory mediators as evidenced by their ability to inhibit production of pro-inflammatory mediators, to induce expression of protective heme oxygenase-1 and to promote relaxation of blood vessels[146]. Most notably, atherosclerosis prone ApoE mice administered nitro-oleic acid were protected from tissue oxidation, foam cell formation, and overall inflammation[144d]. Mechanistically, this class of bioactive lipid can protect via multiple mechanisms including upregulation of PPARγ and Nrf2 as well as downregulation of pro-inflammatory NFκB[147]. Importantly, recent evidence suggests that activation of these pathways exhibits specificity for the chain length and positional substitution of the nitro group[147a]. Although a large body of evidence supports the paradigm that nitro-fatty acids are anti-inflammatory and protective, many unresolved and controversial issues remain.

Integrally important to the progress and advancement of nitro-fatty acid research is the ability to sensitively and accurately measure these species in plasma and tissues. Even though nitro-fatty acids have been studied for over a decade, there still is no universally agreed upon method to extract, identify, and quantitate these species. Due to technological limitations explained in more detail below, relatively basic problems, such as the identification and quantification of free nitro-fatty acid levels in plasma, remain unresolved. For example, depending on what extraction and analysis techniques were utilized, published baseline levels of nitro-oleic acid have ranged from pM to nM concentrations, and no true consensus exists if the observed levels are skewed by artifacts created during sample preparation[226].

Current analytical limitations concerning nitro-fatty acid detection

To date, published measurements of nitro-fatty acids have used electrospray ionization tandem mass spectrometry using triple quadrupole instruments operated in selected ion monitoring mode. A common problem with this approach for free fatty acids is that these species only ionize in negative mode which can result in skewed data due to the acidic conditions necessary for reverse phase chromatography. Nitro-fatty acids do ionize in negative mode but the diagnostic nitrite product ion formed is not truly conclusive of the parent nitro-fatty acid species. An additional issue is that because of the chemical diversity of fatty acids in biological samples, theoretically, these could contain multiple nitro-fatty acid species (or their isotopomers) at every mass unit which traditional quadrupole instruments could misconstrue. Although some of the published work in this field likely provides convincing evidence for the presence of particular nitro-fatty acids in biological samples, these described concerns probably account for much of the uncertainty and confusion in the field regarding the spectrum and absolute levels of nitro-fatty acids present in biological samples. To address these issues we adapted a chemical derivatization approach used in conjunction with high resolution tandem mass spectrometry using a quadrupole time of flight (TOF) instrument to provide a sensitive and specific way to profile and quantitate nitro-fatty acids.

As explained above, a second concern is that nitro-fatty acid parent ions may not be selected specifically at the resolution of quadrupole mass analyzers. To circumvent this problem we next developed methods for analysis of methyl pyridinium derivatives of nitro-fatty acids using our AB Sciex 5600 quadrupole TOF mass spectrometer. In brief, these methods allow us to identify the ions of the parent nitro-fatty acid derivative at high resolution and confirm their identities by other state of the art LC/MS/MS techniques such as mass defect and isotopomer ratio pattern analyses. Further identification of these species is then accomplished by generating high resolution product ion spectra which can help to confirm the presence of the nitronium ion that is diagnostic of nitro-fatty acids.

Nitro-fatty acids are anti-inflammatory and anti-atherosclerotic lipid species that hold much clinical promise, however, due to technological limitations, multiple controversial issues remain unsolved. This proposed study will (1) validate an effective, specific and sensitive nitro-fatty acid extraction procedure; (2) use chemical derivatization HR electrospray ionization tandem mass spectrometry and stable isotope dilution to profile and quantitate nitro-fatty acids in biological samples.

A.3 Materials and methods:

A.3.1 Animal handling and use

Animal procedures were conducted under the approval of the University Committee on Use and Care of Animals at the University of Kentucky and conform to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health. The animals used in this study, C57BL/6J mice, were obtained from the Jackson Laboratory. Mice were injected with 2 nanomoles/gram mouse of 10-nitro-oleate for 10 minutes or vehicle and plasma was collected via cardiac puncture.

A.3.2 Mass spectrometric analysis of nitro-oleate

Quantification of Nitro-oleate in plasma was conducted by high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using an ABSciex 5600 “Triple TOF” hybrid quadrupole time of flight mass spectrometer. 9 and 10-nitro-octadecenoic acid was purchased from Cayman Chemical. Quantification of nitro-oleate in plasma was determined by calibration curve of infused nitro-oleate standard and extraction recovery was normalized to C17 fatty acid internal standard. Plasma (20 µl) were extracted with 80 µl of cold acetonitrile in the presence of C17 fatty acid internal standard (1µM). Samples were vortexed, centrifuged at 14000g 4ºC, for 10 min and supernatant transferred directly into vials to be analyzed by HPLC-MSMS. Nitro FA were measured either in the negative mode or as methyl pyridinium derivatives in positive mode. Samples were derivatized with 2-bromo-1-methylpyridinium iodide (BMP) and 3-carbinol-1-methylpyridinium iodide (CMP), forming 3-acyloxymethyl-1-methylpyridinium iodide (AMMP). Samples were dried, reconstituted in 99/1 methanol/water containing 0.1% ammonium formate and 0.5% formic acid, and analyzed using the ABSciex 5600 instrument. Underivatized Nitro-fatty acids in negative mode were resolved using a Agilent Eclipse XDB C8 column, 5 µM, 4.6 X 150 mm with water + 0.05% formic acid as solvent A and / 95/5- acetonitrile/ water+ 0.05% formic as solvent B. AMMP derivatized nitro-FAs were resolved using a 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) (A) & 99/1of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) (B) solvent system.

HR product ion spectrum of (Nitrooleate, m/z 326.233 M-H-) in the negative mode resulted in product ions of 45.998 and 170.155, which are representative of nitro-oleate. HR MRM scans of 433.2 (AMMP-Nitrooleate) in the positive mode resulted in product ions of m/z 107.07, 122.06, and 386.31 which are derived from AMMP-nitro-oleate.

A.3.3 Acidic chloroform lipid extraction

Ice cold MeOH was added to chloroform and 0.1M HCl. 50 µL of plasma was added to each sample vial along with 50 µL of 1 µM internal standard. Too separate phases, CHCl3 and 1.3 ml 0.1M HCl were added. The lower phase was transferred to 4ml screw cap vial using a pasteur pipette. This was volume was evaporate to dryness under N_2 using N-evap in fume hood and subsequently suspended in 100 µL methanol or derivatized as described above.

A.4 Results

A.4.1 Nitro-oleic acid identification in positive mode is more efficient due to chemical derivatization.

We developed methods for detection and quantitation of nitro-oleic and other nitro-fatty acids using both negative and positive analysis modes using HPLC electrospray ionization tandem high resolution mass spectroscopy. Fragmentation of nitro-oleic acid resulted in ions representing loss of NO_2 (45.99), parent nitro-oleic acid (326.23), and others (Fig. A.1). For positive mode analysis, fatty acids were extracted from plasma and subsequently chemically derivatized to methylpyridinium derivatives. Fragmentation of AMMP-nitro-oleic acid resulted in a TOF product ion scan illustrating ions representing AMMP-nitro-oleic minus loss of NO_2 (386.31), parent AMMP-nitro-oleic acid (433.31), and others (Fig. A.2). The use of derivatization and positive ion mode resulted in approximately a 500 fold enhancement of sensitivity compared to negative mode analysis.

A.4.2 Confirmation of analytical methods using mice exogenously administered nitro-oleic acid.

To test our analytical methodologies in an *in vivo* system, mice were injected with 2 nanomoles/gram mouse nitro-oleic acid and were sacrificed 10 minutes after injection. Blood plasma was collected at this early time point due to nitro-fatty acids' propensity to

adduct to nucleophilic proteins and endogenous glutathione. Nitro-oleic acid was identified both in negative and positive mode (A.4, 5). We were able to detect increases in nitro-oleic acid in mouse plasma following intra peritoneal administration of a single dose (Fig. A.6).

A.4.3 Comparison of two fatty acid extraction procedures

To identify possible alternative extraction procedures that may be more efficient than the acetonitrile extraction commonly used, a secondary extraction procedure was attempted. Use of the acidified chloroform extraction resulted in approximately a 4 fold increase in observable nitro-oleic acid from mouse plasma (Fig. A.7). It is unknown however if the chloroform extraction is more efficient, or produces additional artifact nitro-oleic acid due to the acidified nature of the procedure.

A.5 Discussion

Unsaturated fatty acids and notably certain polyunsaturated fatty acids exhibit beneficial effects in clinical settings and experimental models of cardiovascular and metabolic disease, in part through mechanisms that involve attenuation of oxidative stress and inflammation. It is widely hypothesized that these effects involve enzymatic or non-enzymatic conversion of unsaturated fatty acids to biologically active metabolites. Inconsistencies and/or inter individual variation in formation of these active metabolites may account for variable observations that have plagued this field. Accordingly, definitive identification and quantitation of bioactive fatty acid derivatives is essential for both evaluating the beneficial effects of these nutrients and identifying biological markers to monitor the therapeutic efficacy of fatty acid administration. Nitro-fatty acids are a class of fatty acid metabolites that are formed by chemical nitration of the double bonds of unsaturated fatty acids. Several laboratories have shown that nitro-derivatives of some unsaturated fatty acids can decrease inflammation by down regulation of pro-inflammatory mediators such as NFκB and by activating the antioxidant master controller Nrf2. In mouse models, exogenously supplied nitro-fatty acids decrease systemic oxidative stress and inflammation suggesting that these could be biologically relevant mediators. However, the role of endogenously generated nitro-fatty acids as protective mediators of the actions of dietary unsaturated fatty acids is less clear, in large part due to inherent limitations of currently used analytical approaches for the detection and quantitation of free and esterified nitro-fatty acids. To address these issues we have

developed sensitive, quantitative, and specific methods for analysis of nitro-fatty acids using chemical derivatization and high resolution (HR) tandem mass spectrometry. With these methods in hand we are now in a position to investigate nitro-fatty acid metabolism *in vivo* and evaluate the relationship of nitro-fatty acid formation, vascular inflammation, and toxicant modulation.

The capability to identify and quantitate these and other bioactive lipid species opens up many possible future directions. We have begun to profile levels of nitro-fatty acids in humans and hope to identify previously un-discovered metabolites including nitro-DHA and nitro-EPA fatty acids. PUFAs are known to form multiple anti-inflammatroy bioactive metabolites such as E and D-series resolvins, and the formation of nitro-EPA or nitro-DHA may also be beneficial[127]. It will also be interesting to gain a better understanding of the endogenous control mechanisms of nitro-fatty acid formation. One possible mechanism could be the upregulation eNOS or related NOS enzymes.

Endothelial NOS activation is known to increase cellular nitric oxide levels and is integral to proper cardiovascular function. Normally inhibited by the caveolae associated protein Caveolin-1 (Cav-1), eNOS activation can lead to improved blood pressure and decreased atherosclerotic risk. Endothelial nitric oxide synthase generates nitric oxide which is a key player in vasodilation, thrombosis and cell growth, and decreased activation of eNOS can contribute to hypertension and atherosclerosis [227]. Normally inhibited within caveolae by Cav-1, eNOS can be activated by multiple signaling cascades and molecules such as PI3 kinase, calcium, HDL and estrogen[227]. Activation of eNOS leads to a conversion of L-arginine to L-citrulline which results in the formation of diffusible nitric oxide. If nitric oxide interacts with cellular reactive oxygen species, then nitro-fatty acids are likely to be formed[144b]. Activation of eNOS leads to a greater abundance of nitric oxide and may increase the probability that unsaturated fatty acids become nitrated. Not surprisingly, mice lacking inhibitory Cav-1 exhibit increased eNOS activation, higher levels of nitric oxide, and protection from inflammation and atherosclerosis. Endothelial nitric oxide synthase can be activated during inflammatory responses which may lead to higher levels of anti-inflammatory nitro-fatty acids. A biological system may utilize nitro-fatty acid formation as a mechanism to keep harmful inflammation in-check. It would be interesting to examine the levels of nitro-fatty acids before and after exposure to environmental pollutants or other stressors,

and also determine if upregulation of eNOS or related enzymes prior to the exposure would be protective. Interestingly, multiple lifestyle modifications such as exercise[228] or nutritional modulation have been shown to alter eNOS signaling which may be prime candidates to increase the levels of nitrated fatty acid species.

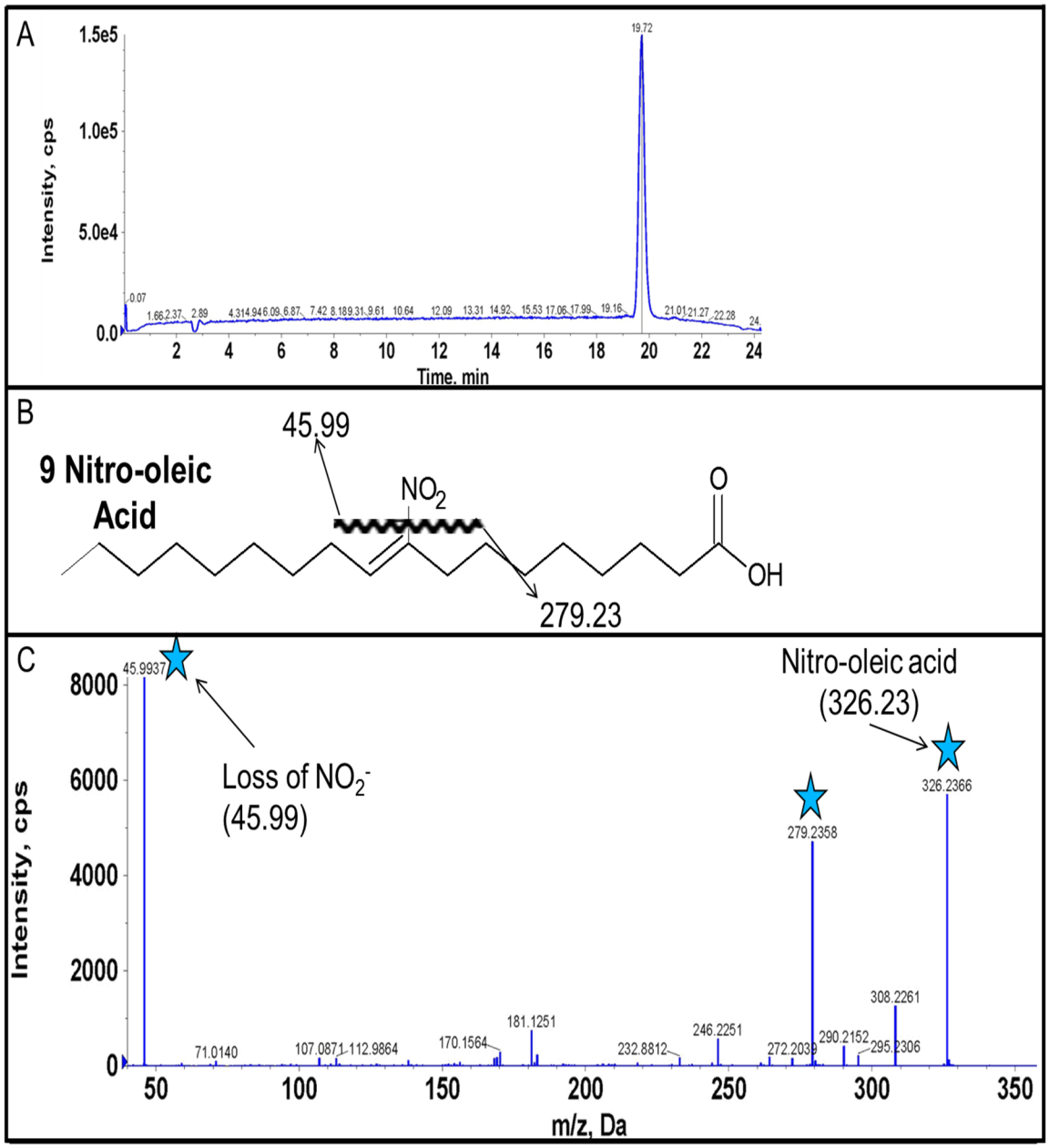

Figure A.1. HR-LC/MS/MS analysis of nitro-oleic acid. A. Extracted ion chromatogram showing the ability to detect nitro-oleic acid by infusing standards. B. Fragmentation pattern of 9 nitro-oleic acid. C. TOF Product ion spectra of nitro-oleic acid. Stars delineate observed ions corresponding to identified fragment ions.

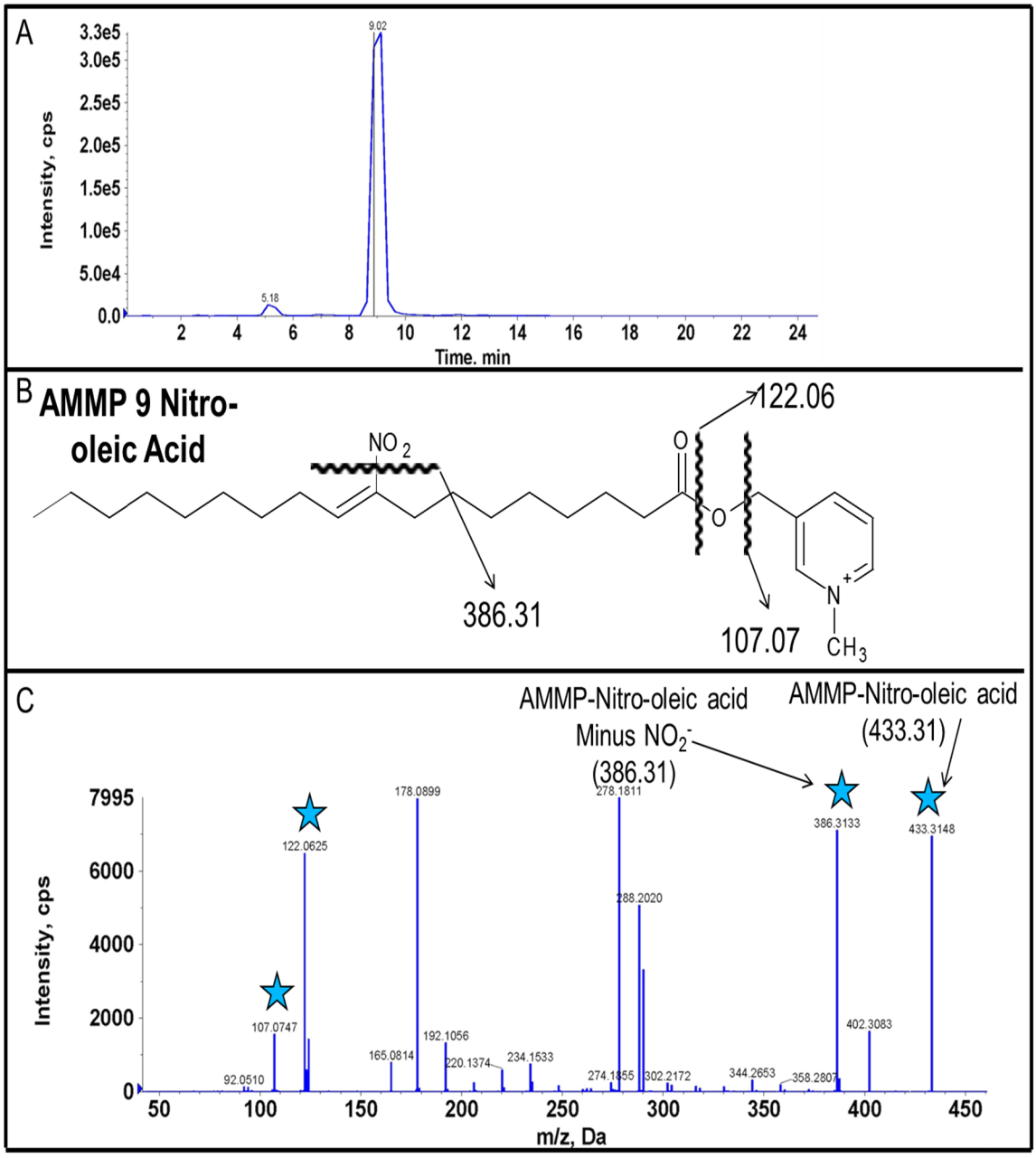

Figure A.2. HR-LC/MS/MS analysis of AMMP-derivatized- 9 nitro-oleic acid. A. Extracted ion chromatogram showing the ability to quantitate AMMP-nitro-oleic acid. B. Fragmentation pattern of AMMP-9 nitro-oleic acid. C. TOF Product ion spectra of AMMP-nitro-oleic acid. Stars delineate observed ions corresponding to identified fragment ions.

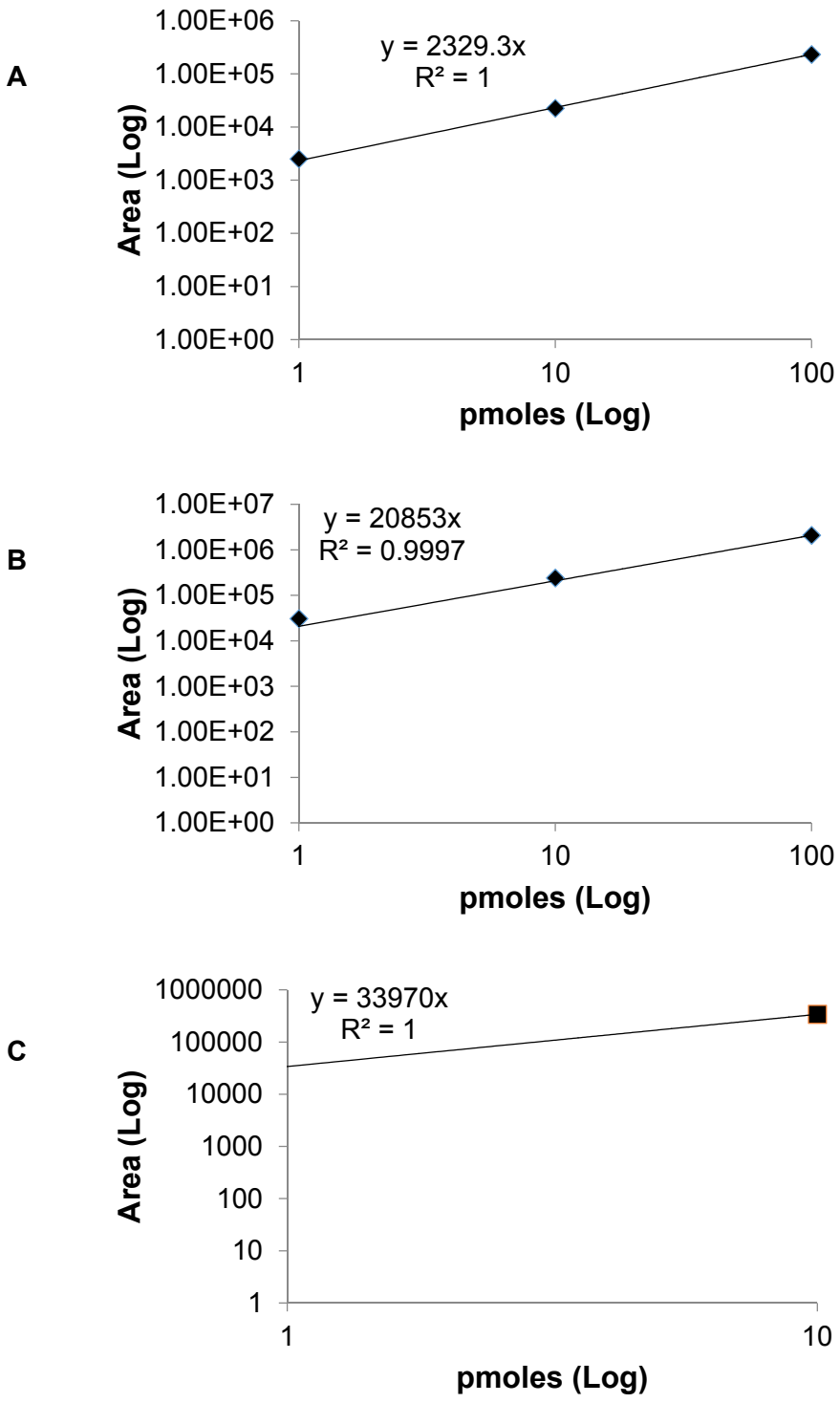

Figure A.3. Quantitation of nitro-oleate using high resolution methodologies. A. Concentration curve for nitro-oleate standard. B. Concentration curve for AMMP-nitro-oleate. C. Concentration curve for AMMP-C17 fatty acid internal standard.

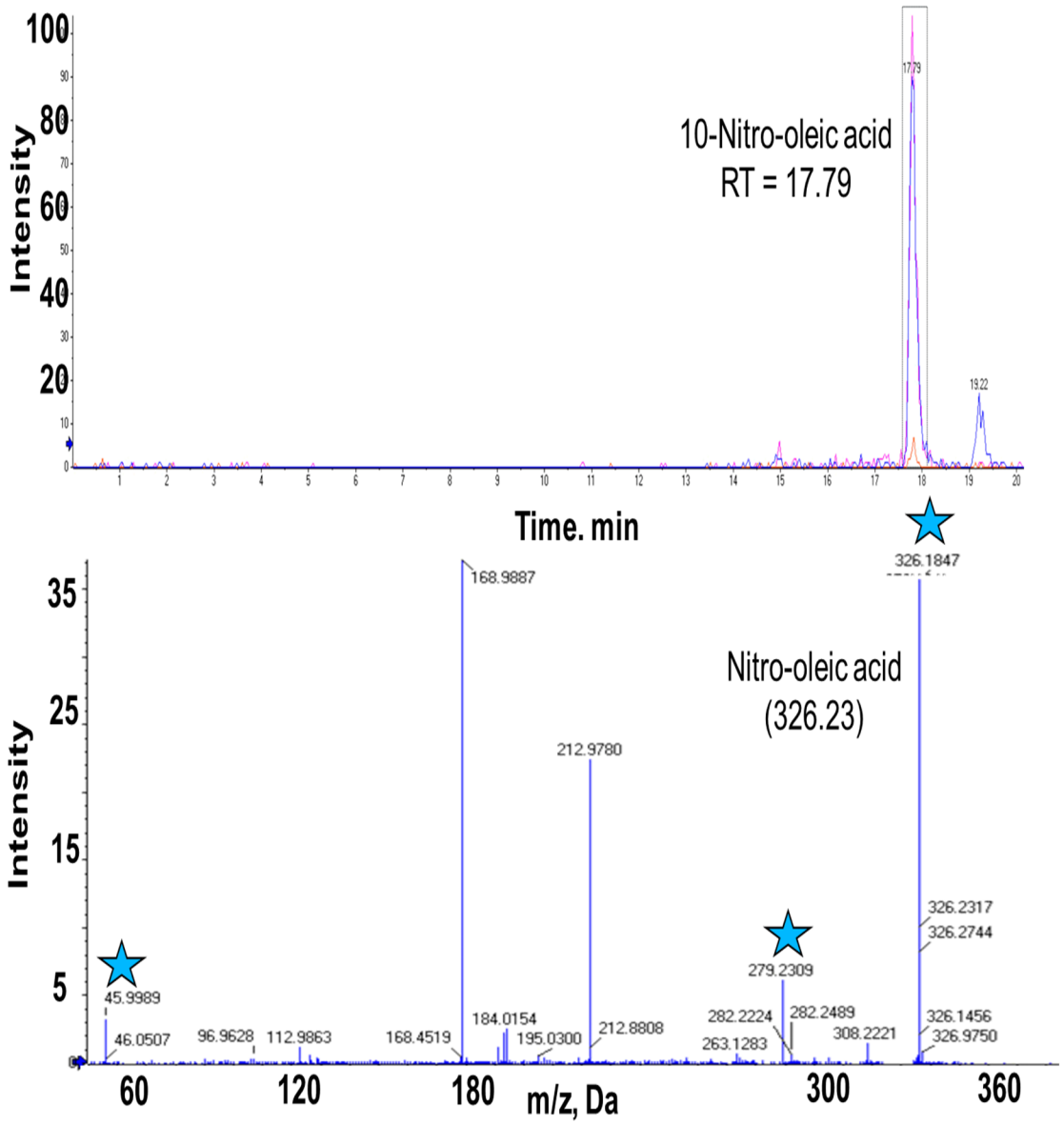

Figure A.4:.Methodologies confirmed using mice exogenously injected with nitro-oleate (negative mode) A**:** HR-LC/MS/MS analysis of nitro-oleic acid in negative ionization mode from plasma obtained from mice injected for 10 minutes post administration of 10-Nitro-oleate. No nitro-oleate was observed in vehicle treated mice.

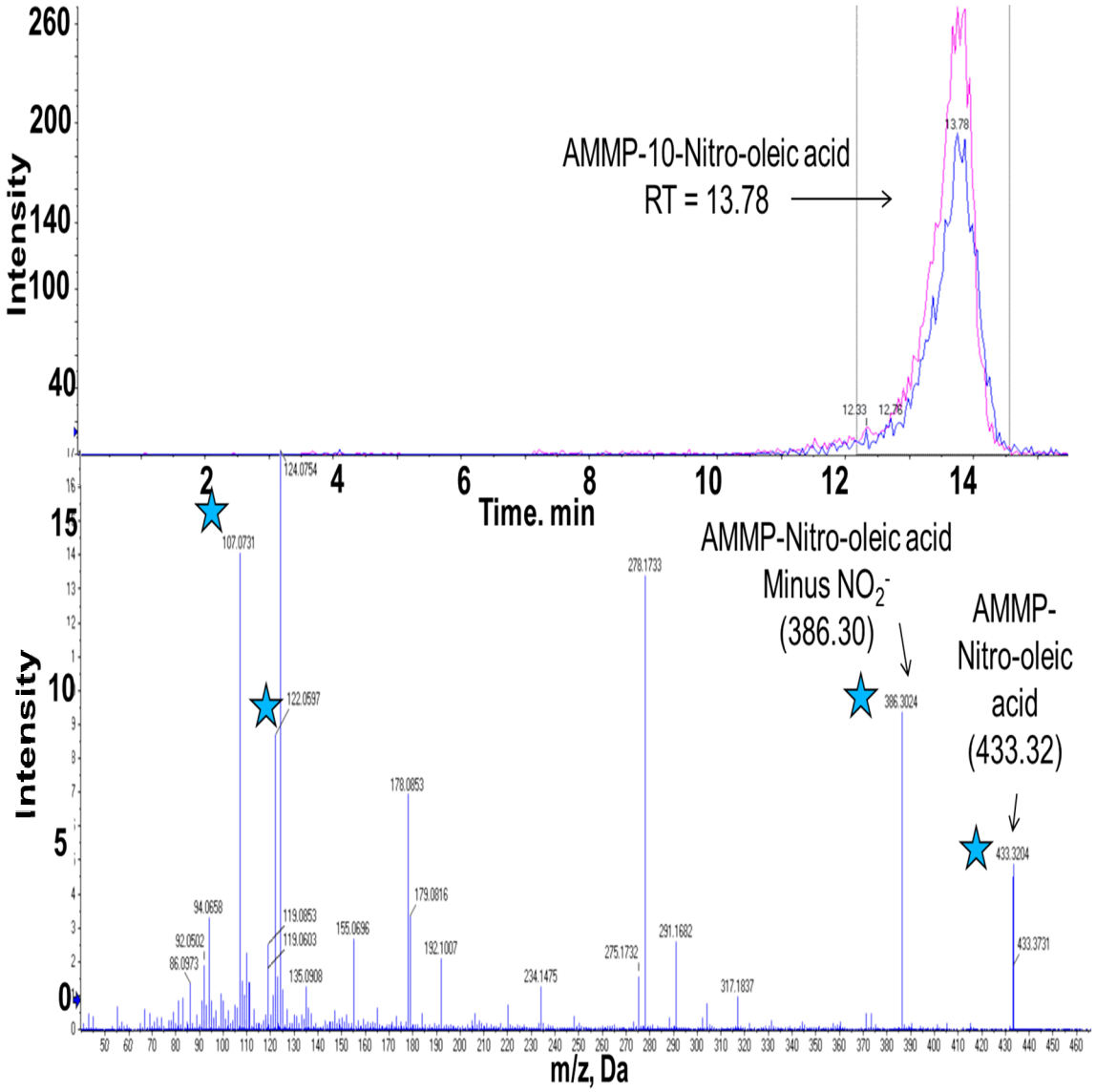

Figure A.5. Methodologies *confirmed using mice exogenously injected with nitro*-oleate (positive mode) HR-LC/MS/MS analysis of AMMP-10-nitro-oleic acid in positive ionization mode from plasma from mice injected for 10 minutes with 10-Nitro-Oleate. AMMP-10-nitro-oleic acid was observed in both control and exogenously administered mice, but to a higher degree in mice injected for 10 minutes with nitro-oleate.

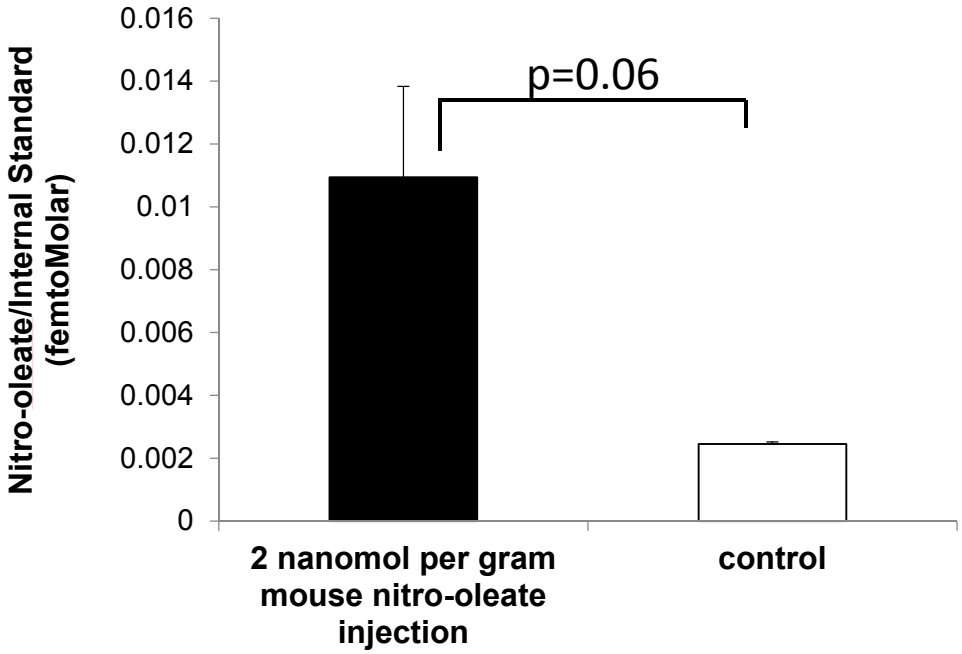

Figure A.6. Concentration of nitro-oleate in mice exogenously administered nitro-oleate. Mice were I.P. injected with 2 nano mole per gram of mouse and sacrificed 10 minutes post injection. Plasma concentrations were determined using AMMP-derivatization technique and positive mode high resolution analysis.

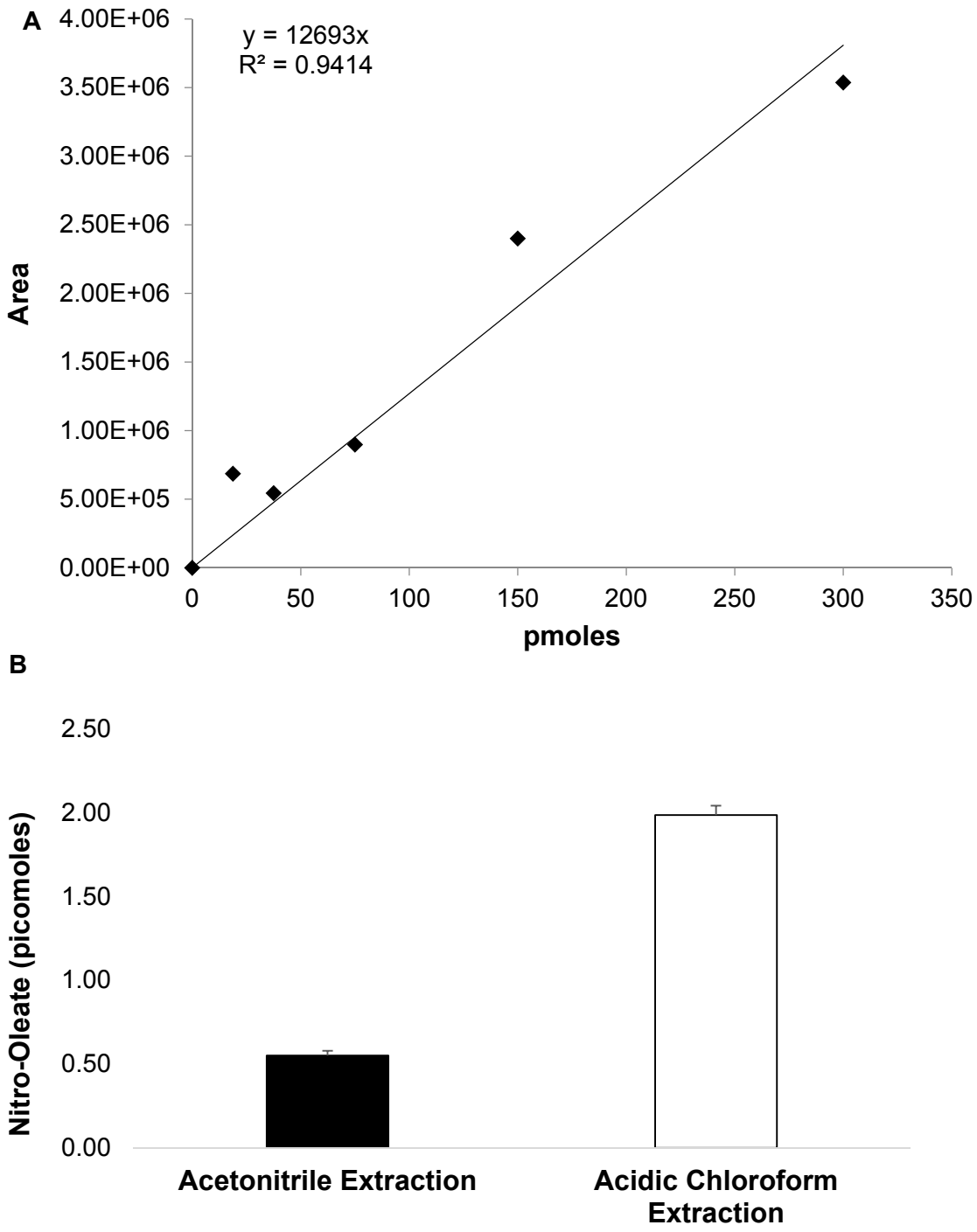

Figure A.7. Comparison of nitro-fatty acid extraction procedures. A. Standard curve of AMMP-nitro oleic acid used for quantitation. B. Two extraction procedures were compared (see materials and methods) and the acidic chloroform extraction resulted in ~4 fold increase in observable nitro oleic acid in plasma.

Appendix B. Lab protocols

1. mRNA related protocols:

Trizol Harvest

- Warm up Trizol reagent in water bath for about 20-30 minutes prior to harvest
- **For 6 well plates (*in vitro* experiments)**
    - Wash each well once with 1 mL cold PBS and aspirate
    - Add 600 µL Trizol to each well and scrape with reusable cell scraper
    - For each well, pipet up and down 7 times to homogenize liquid and transfer to new autoclaved 1.7 mL Eppendorf tube.
    - Place in -80 freezer overnight.
- **For mouse tissues (e.g., liver and hearts)**
    - Harvest tissues and place in *RNA Later* solution (enough solution to cover tissue)
    - Place in 4 degree fridge for short term (few days) or -20 freezer for longer
    - In 2 mL colored tubes (round bottomed) add 1 mL Trizol reagent, 50-100 mg tissue of interest, and 2 autoclaved homogenizer balls (ball bearings).
    - Use the Cassis lab's homogenizer per their instructions for your tissue of interest.
    - Remove ball bearings with "super magnet" and freeze tubes at -80 overnight.

mRNA isolation:

- **For 6 well plates (*in vitro* experiments)**
    - Let tubes thaw at room temperature in hood.
    - Add 120 µL Chloroform to each tube and calmly shake for 15 seconds. Incubate at room temperature for 5 minutes
    - Centrifuge tubes at 12,000 X g for 15 minutes at 4 degrees. Upon completion you should see distinct phase separation.
    - Using a p200 pipet, **carefully** collect the clear phase without disrupting the cloudy or red phases (I pipet 150 µL twice in to a new tube).
    - Add 300 µL of isopropyl alcohol to each tube and invert each tube just twice to mix.
    - Centrifuge at 12,000 x g for 10 minutes at 4 degrees. Hopefully you can see a pellet.
    - Remove the supernatant by decanting and then place each tube inverted on kim wipe to remove excess isopropyl alcohol
    - Wash the pellet with 600 µL of 75% ethanol solution (made up with rnase/dnase free water). Vortex briefly.
    - Centrifuge at 7,500 x g at 4 degrees for only 5 minutes.
    - Decant the ethanol and then place each tube inverted on Kim wipe to remove excess. Cap tubes and do a quick spin to collect all remaining

liquid. Using small pipet, carefully remove all remaining liquid without sucking up pellet. Dry pellet for 5 minutes.
    - Add 12 µL of dnase/rnase free water and pipet up and down 3 times to dissolve pellet.
    - Quantify RNA concentration using the nanodrop down the hall.
- **For mouse tissues**
    - Let tubes thaw at room temperature in hood.
    - To remove tissues, centrifuge at 12,000 x g for 10 minutes at 4 degrees. Pipet supernatant to new tube.
    - Add 200 µL Chloroform to each tube and calmly shake for 15 seconds. Incubate at room temperature for 5 minutes
    - Centrifuge tubes at 12,000 X g for 15 minutes at 4 degrees. Upon completion you should see distinct phase separation.
    - Using a p200 pipet, **carefully** collect the clear phase without disrupting the cloudy or red phases (I pipet 210 µL multiple times in to a new tube).
    - Add 500 µL of isopropyl alcohol to each tube and invert each tube just twice to mix.
    - Centrifuge at 12,000 x g for 10 minutes at 4 degrees. Hopefully you can see a pellet.
    - Remove the supernatant by decanting and then place each tube inverted on kim wipe to remove excess isopropyl alcohol
    - Wash the pellet with 1 mL of 75% ethanol solution (made up with rnase/dnase free water). Vortex briefly.
    - Centrifuge at 7,500 x g at 4 degrees for only 5 minutes.
    - Decant the ethanol and then place each tube inverted on Kim wipe to remove excess. Cap tubes and do a quick spin to collect all remaining liquid. Using small pipet, carefully remove all remaining liquid without sucking up pellet. Dry pellet for 5 minutes.
    - Add 50-250 µL of dnase/rnase free water depending on the size of pellet and pipet up and down 3 times to dissolve pellet. If not sure always be conservative and then check via Spec. Can always dilute more in front of the nanodrop.
    - Quantify RNA concentration using the nanodrop down the hall.

Reverse Transcription protocol: AMV reverse transcription system (Promega)

- Make 1-2 µg cDNA per sample depending on your spec values.
    - Use “Spec spreadsheet” to determine how much mRNA and water is needed to have 2 ug in 9.9 µL total volume. Spin down using small table top centrifuge.
    - Place PCR tubes in thermo cycler and run “LEI/**HEAT”** program (~10 minutes).
    - Make Master Mix for all samples in 1.7 mL Eppendorf. If you have 20 samples, make the mix for 22 or 23 samples. See below. All values are micro liters

		x40
MgCl2	4	160
RT 10x buffer	2	80
dNTP	2	80
RNAsin	0.5	20
Random Primers	1	40
AMV RT	0.6	24
Total	10.1	404

- Add 10.1 µL of Master Mix to each tube containing mRNA and spin with small centrifuge.
- Place tubes in thermo cycler and run "LEI/**RT**" (~90 minutes).
- When finished, dilute 1:10 with dnase/rnase water.

Real Time PCR

*Every plate contains 96 wells. You must always run a control such as Actin or GAPDH on **EVERY** plate. Run every sample with duplicates or triplicates.

Set up plate as shown in template.

- Add 2 µl of cDNA per well.
- Make a master mix for each gene of interest (e.g., actin gets one tube and AhR gets a second Eppendorf).
  - If you have 48 rxns. Make master mix for 51 or 52:
    - 10 µl of Cyber Green reagent
    - 5.6 µl of dnase/rnase free water
    - 1.2 µl Forward primer of interest
    - 1.2 µl Reverse primer of interest
  - Seal tightly with plastic plate sealer and centrifuge for 5 minutes (800 rpm).

2. Isolation of mouse lung endothelial cells

DAY 1:

Lung tissue isolation:

- Use lung tissue from **2-3 mice per isolation**

- Record the weight, #, genotype, and punch of each mouse
- Anaesthetize mice using Isoflurane and euthanize by cervical dislocation
- Sterilize skin with 70% ethanol and remove underlying tissues (the heart should be beating)
- Locate and cut the right atrium flap at the base of the heart
- Insert an 18 gauge needle (connected to a 50ml syringe filled with PBS) into the left ventricle and perfuse SLOWLY with (5ml of pre-warmed heparinized PBS with heparin to prevent clotting (**3ml heparin/500ml PBS; prepared on same day**)
- If perfusion is done properly:
    - Perfused fluid should flow out of the right atrium
    - Liver color will change from dark red to light brown
    - Fast perfusion may cause damage to blood vessels and endothelial cells
- Isolate lungs using forceps and immerse into pre-chilled **0.1% BSA/DMEM** (High glucose) solution until ready for the next step (up to 6 hrs)

Homogenization:

- Mince tissue (using a blade) into 1.1-2mm pieces and place in a 50ml conical tube filled with pre-warmed culture media (**prepared on the same day**) containing
    - DMEM (**45ml**)
    - 1% fatty acid free BSA (**5ml of 10% stock solution**)
    - Type II Collagenase- 2.0mg/ml (**100mg**)
    - Dispase II- 4mg/ml (**200mg**)
- Cap tubes tightly and wrap with parafilm; shake horizontally at 37°C for **exactly** 1hr at 225rpm
- Filter the homogenized tissue through a stainless mesh into a pre-labeled 50ml conical tube
- Centrifuge the filtered tissue at 37°C for 8min at 1000rpm
- Discard the supernatant using suction and resuspend the pellet in 1ml of lung endothelial cell growth media (LECM) containing the following:
    - DMEM (**387ml**)
    - 20% FBS (**100ml**)
    - 100ug/ml heparin (**3ml**)
    - P/S antibiotics (**10ml**)
- Aliquot LECM into pre-labeled 50ml conical tubes and store in the 4°C refrigerator
- Thaw and add 1ml of Endothelial Cell Growth Supplement (ECGS stored in -80 freezer) to each 50ml aliquot to prepare complete LECM prior to use (**on the same day and up to 1wk**)

Plating cells (P0):

- Coat 35mm plates (preferably in 6 well plates) with 0.5% gelatin (prepared fresh and stored in 15ml aliquots in the 4°C refrigerator) and incubate under UV light for 1hr (**on the same day**)
- Label the plate clearly with the date, generation (P_0) and genotype for each well
- Add 1ml of complete LECM to each well and pre-warm in the 37°C incubator
- Resuspend each pellet in 1ml of complete LECM and transfer to the corresponding well in the 6-well plate (final volume of 2ml LECM/well)
- Swirl gently to spread cells homogenously and incubate at 37°C, 5% CO_2 overnight

DAY 2:

Washing cells:

- Wash P0 plates 5 times with pre-warmed PBS to eliminate cellular debris

DAY 3:

Preparing antibody coated bead mix for bead selection:

- Amounts of beads and antibody are scaled to one 35mm plate
- Add **25ul of M450 sheep anti-rat IgG beads** in a 1.5ml eppendorf tube
- Wash the beads:
    - Add 1ml of PBS to the tube, and gently invert the tube 3 times to suspend the beads
    - Place the tube in the Dynacare magnet, gently invert the tube 3 times
    - After beads adhere to the wall of the tube, aspirate PBS using suction
    - Repeat the steps described above 3 times
- Resuspend the beads with 1ml PBS, add **2.5ul of rat anti-mouse ICAM-2 or CD31 PECAM antibody**
- Cap the tube tightly and incubate for 1hr/overnight on the nutator under UV light
- Pull the ICAM-2 antibody bound beads using the magnet and wash 3 times with 1ml LECM
- Resuspend the ICAM-2 antibody bound beads in 1ml of LECM and incubate at 37° until P0 plates are ready for bead selection

First bead purification of endothelial cells (P1):

- Wash P0 endothelial cells (in 35mm plates/wells) 3 times with pre-warmed PBS
- Aspirate PBS and add 1ml of LECM
- Add 1 ml of the antibody-bound beads suspended in LECM
- Incubate the plates at 37°C, 5% CO_2 for 1hr, and gently shake every 15min
- Wash plates with 2-3 times with PBS, add 1ml trypsin/35mm plate, and incubate at 37°C, 5% CO_2 for 5min or until cells are afloat
- When cells are dissociated, add 0.5ml of LECM to stop trypsin reaction

- Transfer the contents of each 35mm plate to a pre-labeled 1.5ml eppendorf tubes and apply to the Dynal magnetic separator
- Wash bead-bound endothelial cells 4-5 times with DMEM/0.1% FBS
- Resuspend cells in ECGM and count the cells using the hemacytometer (described below)
- Seed 2.0-4.0x10^5 cells in a final volume of 1ml ECGM/35mm plate
- Shake gently to mix and incubate at 37°C, 5% CO_2 overnight
- Label plates with date, phenotype, Passage number (P1)
- Change LECM every 2-3 days

3. Silencing protocol

- Label 3, 15mL Falcon tubes
    - Cav-1, Ctl, Gene Silencer

Tube			X10
	siRNA diluent	24	240
1	optimem	15	150
	ctl siRNA	1	10
2	cav-1 (2)	0.5	5
	cav-1 (5)	0.5	5
			X20
3	Gene Silencer	5	100
	Optimem	25	500

- Add dilluent, Optimem, and appropriate siRNA to tubes 1 & 2
    - Mix by flicking tube ~15 times
- Add Gene silencer and Optimem to tube 3
    - Mix by hand gently by slightly inverting tube.
- INCUBATE ALL 3 TUBES FOR 5 MINUTES
- Take half of volume in Gene Silencer tube (tube 3) and pipet directly into tube 1. Other half to tube 2.
    - Mix tube 1 & 2 gently by hand by slightly inverting tube
- INCUBATE FOR 20 MINUTES
    - After 10 minutes prepare cells for transfection by washing twice with Optimem.
        - 1 mL for 6 well plate & 0.5 mL for 12 well plate
- After 15 minutes, dilute tubes 1 & 2 with Optimem up to total volume necessary for amount of wells (Use graduations on Falcon Tube).
    - For Example : 10mls total in each tube for 1, 6 well plate and 8 wells of a single 12 well plate

- Add 1 mL per well in 6 well plate, and 0.5 mL per well in 12 well.
    - DROP BY DROP using large serological pipet
- After 4 hours add 20% FBS M199 without antibiotics
- Incubate for 48 hours before continuing with experiments.

4. Acidic chloroform lipid extraction

- Need 8 ml borosilicate glass tubes with Teflon cap
- Add 2ml ice cold MeOH and add 1ml $CHCl_3$
- Add 450 µL 0.1M HCl to tube
- Add 50 µL of plasma to each sample
- Add 50 µL of 1 µM internal standard to each tube
- Vortex for 5 minutes (works best if you have a multi tube vortexer)
- Place on ice or at 4 degrees for at least 1 hour
- Add 1 ml $CHCl_3$ and 1.3 ml 0.1M HCl to separate phases
- Vortex for 5 minutes
- Centrifuge at >3,000 x g for 10 minutes
- Transfer lower phase to 4ml screw cap vial using a pasteur pipette. Take care to avoid the protein interface and upper phase. Leave behind ~50 microliters if needed (don't worry about being more accurate than this- we have an internal standard to correct for recovery)
- Evaporate to dryness under N_2 using N-evap in fume hood
- Resuspend in 100 µL methanol
- Transfer to autosampler vial with glass or polypropylene insert for LC MS analysis

References

1. Martin-Timon, I.; Sevillano-Collantes, C.; Segura-Galindo, A.; Del Canizo-Gomez, F. J., Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World journal of diabetes* **2014,** *5* (4), 444-70.
2. Pober, J. S.; Sessa, W. C., Evolving functions of endothelial cells in inflammation. *Nature reviews. Immunology* **2007,** *7* (10), 803-15.
3. Libby, P., Inflammation in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **2012,** *32* (9), 2045-51.
4. Bobryshev, Y. V., Monocyte recruitment and foam cell formation in atherosclerosis. *Micron* **2006,** *37* (3), 208-22.
5. Sumpio, B. E.; Riley, J. T.; Dardik, A., Cells in focus: endothelial cell. *The international journal of biochemistry & cell biology* **2002,** *34* (12), 1508-12.
6. Brown, A. A.; Hu, F. B., Dietary modulation of endothelial function: implications for cardiovascular disease. *Am J Clin Nutr* **2001,** *73* (4), 673-86.
7. Hennig, B.; Meerarani, P.; Ramadass, P.; Watkins, B. A.; Toborek, M., Fatty acid-mediated activation of vascular endothelial cells. *Metabolism: clinical and experimental* **2000,** *49* (8), 1006-13.
8. Ramsden, C. E.; Ringel, A.; Feldstein, A. E.; Taha, A. Y.; MacIntosh, B. A.; Hibbeln, J. R.; Majchrzak-Hong, S. F.; Faurot, K. R.; Rapoport, S. I.; Cheon, Y.; Chung, Y. M.; Berk, M.; Mann, J. D., Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. *Prostaglandins, leukotrienes, and essential fatty acids* **2012,** *87* (4-5), 135-41.
9. Tachibana, H.; Koga, K.; Fujimura, Y.; Yamada, K., A receptor for green tea polyphenol EGCG. *Nature structural & molecular biology* **2004,** *11* (4), 380-1.
10. Gallo, M. V.; Schell, L. M.; DeCaprio, A. P.; Jacobs, A., Levels of persistent organic pollutant and their predictors among young adults. *Chemosphere* **2011,** *83* (10), 1374-82.
11. Baker, N. A.; Karounos, M.; English, V.; Fang, J.; Wei, Y.; Stromberg, A.; Sunkara, M.; Morris, A. J.; Swanson, H. I.; Cassis, L. A., Coplanar polychlorinated biphenyls impair glucose homeostasis in lean C57BL/6 mice and mitigate beneficial effects of weight loss on glucose homeostasis in obese mice. *Environmental health perspectives* **2013,** *121* (1), 105-10.
12. (a) Han, S. G.; Eum, S. Y.; Toborek, M.; Smart, E.; Hennig, B., Polychlorinated biphenyl-induced VCAM-1 expression is attenuated in aortic endothelial cells isolated from caveolin-1 deficient mice. *Toxicology and applied pharmacology* **2010,** *246* (1-2), 74-82; (b) Lim, E. J.; Majkova, Z.; Xu, S.; Bachas, L.; Arzuaga, X.; Smart, E.; Tseng, M. T.; Toborek, M.; Hennig, B., Coplanar polychlorinated biphenyl-induced CYP1A1 is regulated through caveolae signaling in vascular endothelial cells. *Chem Biol Interact* **2008,** *176* (2-3), 71-8; (c) Lim, E. J.; Smart, E. J.; Toborek, M.; Hennig, B., The role of caveolin-1 in PCB77-induced eNOS phosphorylation in human-derived endothelial cells. *American journal of physiology. Heart and circulatory physiology* **2007,** *293* (6), H3340-7.
13. (a) Petriello, M. C.; Newsome, B. J.; Dziubla, T. D.; Hilt, J. Z.; Bhattacharyya, D.; Hennig, B., Modulation of persistent organic pollutant toxicity through nutritional intervention: emerging opportunities in biomedicine and environmental remediation. *The Science of the total environment* **2014,** *491-492*, 11-6; (b) Petriello, M. C.; Newsome, B.;

Hennig, B., Influence of nutrition in PCB-induced vascular inflammation. *Environmental science and pollution research international* **2014,** *21* (10), 6410-8.
14. Arsenescu, V.; Arsenescu, R. I.; King, V.; Swanson, H.; Cassis, L. A., Polychlorinated biphenyl-77 induces adipocyte differentiation and proinflammatory adipokines and promotes obesity and atherosclerosis. *Environmental health perspectives* **2008,** *116* (6), 761-8.
15. Lundqvist, C.; Zuurbier, M.; Leijs, M.; Johansson, C.; Ceccatelli, S.; Saunders, M.; Schoeters, G.; ten Tusscher, G.; Koppe, J. G., The effects of PCBs and dioxins on child health. *Acta paediatrica (Oslo, Norway : 1992). Supplement* **2006,** *95* (453), 55-64.
16. Crinnion, W. J., Polychlorinated biphenyls: persistent pollutants with immunological, neurological, and endocrinological consequences. *Alternative medicine review : a journal of clinical therapeutic* **2011,** *16* (1), 5-13.
17. Kim, M. J.; Marchand, P.; Henegar, C.; Antignac, J. P.; Alili, R.; Poitou, C.; Bouillot, J. L.; Basdevant, A.; Le Bizec, B.; Barouki, R.; Clement, K., Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. *Environmental health perspectives* **2011,** *119* (3), 377-83.
18. Giesy, J. P.; Kannan, K.; Blankenship, A. L.; Jones, P. D.; Hilscherova, K., Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. *Central European journal of public health* **2000,** *8 Suppl*, 43-5.
19. Schulze, P. C.; Lee, R. T., Oxidative stress and atherosclerosis. *Current atherosclerosis reports* **2005,** *7* (3), 242-8.
20. (a) Schlezinger, J. J.; Struntz, W. D.; Goldstone, J. V.; Stegeman, J. J., Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. *Aquatic toxicology* **2006,** *77* (4), 422-32; (b) Schlezinger, J. J.; White, R. D.; Stegeman, J. J., Oxidative inactivation of cytochrome P-450 1A (CYP1A) stimulated by 3,3',4,4'-tetrachlorobiphenyl: production of reactive oxygen by vertebrate CYP1As. *Mol Pharmacol* **1999,** *56* (3), 588-97.
21. Libby, P., Inflammation in atherosclerosis. *Nature* **2002,** *420* (6917), 868-74.
22. Hennig, B.; Meerarani, P.; Slim, R.; Toborek, M.; Daugherty, A.; Silverstone, A. E.; Robertson, L. W., Proinflammatory properties of coplanar PCBs: in vitro and in vivo evidence. *Toxicology and applied pharmacology* **2002,** *181* (3), 174-83.
23. (a) Eske, K.; Newsome, B.; Han, S. G.; Murphy, M.; Bhattacharyya, D.; Hennig, B., PCB 77 dechlorination products modulate pro-inflammatory events in vascular endothelial cells. *Environmental science and pollution research international* **2014,** *21* (10), 6354-64; (b) Majkova, Z.; Smart, E.; Toborek, M.; Hennig, B., Up-regulation of endothelial monocyte chemoattractant protein-1 by coplanar PCB77 is caveolin-1-dependent. *Toxicol Appl Pharmacol* **2009,** *237* (1), 1-7.
24. (a) Uemura, H., [Associations of exposure to dioxins and polychlorinated biphenyls with diabetes: based on epidemiological findings]. *Nihon eiseigaku zasshi. Japanese journal of hygiene* **2012,** *67* (3), 363-74; (b) Carpenter, D., Exposure to Polychlorinated Biphenyls Is Associated With an Increased Risk of Hypertension and Cardiovascular Disease. *Epidemiology* **2011,** *22* (1), S147-S147; (c) Goncharov, A.; Haase, R. F.; Santiago-Rivera, A.; Morse, G.; McCaffrey, R. J.; Rej, R.; Carpenter, D. O., High serum PCBs are associated with elevation of serum lipids and cardiovascular disease in a Native American population. *Environmental research* **2008,** *106* (2), 226-39.
25. (a) Schettgen, T.; Gube, M.; Esser, A.; Alt, A.; Kraus, T., Plasma polychlorinated biphenyls (PCB) levels of workers in a transformer recycling company, their family members, and employees of surrounding companies. *Journal of toxicology and environmental health. Part A* **2012,** *75* (8-10), 414-22; (b) Gustavsson, P.; Hogstedt, C.,

A cohort study of Swedish capacitor manufacturing workers exposed to polychlorinated biphenyls (PCBs). *American journal of industrial medicine* **1997,** *32* (3), 234-9.
26. Butler Walker, J.; Seddon, L.; McMullen, E.; Houseman, J.; Tofflemire, K.; Corriveau, A.; Weber, J. P.; Mills, C.; Smith, S.; Van Oostdam, J., Organochlorine levels in maternal and umbilical cord blood plasma in Arctic Canada. *The Science of the total environment* **2003,** *302* (1-3), 27-52.
27. Hopf, N. B.; Ruder, A. M.; Succop, P., Background levels of polychlorinated biphenyls in the U.S. population. *The Science of the total environment* **2009,** *407* (24), 6109-19.
28. Ha, M. H.; Lee, D. H.; Jacobs, D. R., Association between serum concentrations of persistent organic pollutants and self-reported cardiovascular disease prevalence: results from the National Health and Nutrition Examination Survey, 1999-2002. *Environmental health perspectives* **2007,** *115* (8), 1204-9.
29. Labarthe, D. R.; Briss, P. A., Urinary sodium excretion and cardiovascular disease mortality. *JAMA : the journal of the American Medical Association* **2011,** *306* (10), 1084-5; author reply 1086-7.
30. Organization, W. H., Global status report on noncommunicable diseases 2010. *WHO* **2011**.
31. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H., Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care* **2004,** *27* (5), 1047-53.
32. Tang, M.; Chen, K.; Yang, F.; Liu, W., Exposure to organochlorine pollutants and type 2 diabetes: a systematic review and meta-analysis. *PloS one* **2014,** *9* (10), e85556.
33. Fox, C. S.; Coady, S.; Sorlie, P. D.; D'Agostino, R. B., Sr.; Pencina, M. J.; Vasan, R. S.; Meigs, J. B.; Levy, D.; Savage, P. J., Increasing cardiovascular disease burden due to diabetes mellitus: the Framingham Heart Study. *Circulation* **2007,** *115* (12), 1544-50.
34. Fox, C. S.; Coady, S.; Sorlie, P. D.; Levy, D.; Meigs, J. B.; D'Agostino, R. B., Sr.; Wilson, P. W.; Savage, P. J., Trends in cardiovascular complications of diabetes. *JAMA : the journal of the American Medical Association* **2004,** *292* (20), 2495-9.
35. European Association for Cardiovascular, P.; Rehabilitation; Reiner, Z.; Catapano, A. L.; De Backer, G.; Graham, I.; Taskinen, M. R.; Wiklund, O.; Agewall, S.; Alegria, E.; Chapman, M. J.; Durrington, P.; Erdine, S.; Halcox, J.; Hobbs, R.; Kjekshus, J.; Filardi, P. P.; Riccardi, G.; Storey, R. F.; Wood, D.; Guidelines, E. S. C. C. f. P.; Committees, ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). *European heart journal* **2011,** *32* (14), 1769-818.
36. (a) Airaksinen, R.; Rantakokko, P.; Eriksson, J. G.; Blomstedt, P.; Kajantie, E.; Kiviranta, H., Association between type 2 diabetes and exposure to persistent organic pollutants. *Diabetes care* **2011,** *34* (9), 1972-9; (b) Codru, N.; Schymura, M. J.; Negoita, S.; Rej, R.; Carpenter, D. O., Diabetes in relation to serum levels of polychlorinated biphenyls and chlorinated pesticides in adult Native Americans. *Environmental health perspectives* **2007,** *115* (10), 1442-7; (c) Everett, C. J.; Frithsen, I. L.; Diaz, V. A.; Koopman, R. J.; Simpson, W. M., Jr.; Mainous, A. G., 3rd, Association of a polychlorinated dibenzo-p-dioxin, a polychlorinated biphenyl, and DDT with diabetes in the 1999-2002 National Health and Nutrition Examination Survey. *Environmental research* **2007,** *103* (3), 413-8; (d) Gasull, M.; Pumarega, J.; Tellez-Plaza, M.; Castell, C.; Tresserras, R.; Lee, D. H.; Porta, M., Blood concentrations of persistent organic pollutants and prediabetes and diabetes in the general population of Catalonia. *Environmental science & technology* **2012,** *46* (14), 7799-810; (e) Grandjean, P.;

Henriksen, J. E.; Choi, A. L.; Petersen, M. S.; Dalgard, C.; Nielsen, F.; Weihe, P., Marine food pollutants as a risk factor for hypoinsulinemia and type 2 diabetes. *Epidemiology* **2011,** *22* (3), 410-7; (f) Silverstone, A. E.; Rosenbaum, P. F.; Weinstock, R. S.; Bartell, S. M.; Foushee, H. R.; Shelton, C.; Pavuk, M., Polychlorinated biphenyl (PCB) exposure and diabetes: results from the Anniston Community Health Survey. *Environ Health Perspect* **2012,** *120* (5), 727-32; (g) Vasiliu, O.; Cameron, L.; Gardiner, J.; Deguire, P.; Karmaus, W., Polybrominated biphenyls, polychlorinated biphenyls, body weight, and incidence of adult-onset diabetes mellitus. *Epidemiology* **2006,** *17* (4), 352-9.
37. Arrebola, J. P.; Gonzalez-Jimenez, A.; Fornieles-Gonzalez, C.; Artacho-Cordon, F.; Olea, N.; Escobar-Jimenez, F.; Fernandez-Soto, M. L., Relationship between serum concentrations of persistent organic pollutants and markers of insulin resistance in a cohort of women with a history of gestational diabetes mellitus. *Environmental research* **2015,** *136*, 435-40.
38. American Diabetes, A., Gestational diabetes mellitus. *Diabetes care* **2004,** *27 Suppl 1*, S88-90.
39. Lee, D. H.; Steffes, M. W.; Sjodin, A.; Jones, R. S.; Needham, L. L.; Jacobs, D. R., Jr., Low dose organochlorine pesticides and polychlorinated biphenyls predict obesity, dyslipidemia, and insulin resistance among people free of diabetes. *PloS one* **2011,** *6* (1), e15977.
40. Jensen, T. K.; Timmermann, A. G.; Rossing, L. I.; Ried-Larsen, M.; Grontved, A.; Andersen, L. B.; Dalgaard, C.; Hansen, O. H.; Scheike, T.; Nielsen, F.; Grandjean, P., Polychlorinated biphenyl exposure and glucose metabolism in 9-year-old danish children. *The Journal of clinical endocrinology and metabolism* **2014,** *99* (12), E2643-51.
41. (a) Jorgensen, M. E.; Borch-Johnsen, K.; Bjerregaard, P., A cross-sectional study of the association between persistent organic pollutants and glucose intolerance among Greenland Inuit. *Diabetologia* **2008,** *51* (8), 1416-22; (b) Persky, V.; Piorkowski, J.; Turyk, M.; Freels, S.; Chatterton, R., Jr.; Dimos, J.; Bradlow, H. L.; Chary, L. K.; Burse, V.; Unterman, T.; Sepkovic, D.; McCann, K., Associations of polychlorinated biphenyl exposure and endogenous hormones with diabetes in post-menopausal women previously employed at a capacitor manufacturing plant. *Environmental research* **2011,** *111* (6), 817-24; (c) Persky, V.; Piorkowski, J.; Turyk, M.; Freels, S.; Chatterton, R., Jr.; Dimos, J.; Bradlow, H. L.; Chary, L. K.; Burse, V.; Unterman, T.; Sepkovic, D. W.; McCann, K., Polychlorinated biphenyl exposure, diabetes and endogenous hormones: a cross-sectional study in men previously employed at a capacitor manufacturing plant. *Environmental health : a global access science source* **2012,** *11*, 57.
42. Lee, D. H.; Lee, I. K.; Porta, M.; Steffes, M.; Jacobs, D. R., Jr., Relationship between serum concentrations of persistent organic pollutants and the prevalence of metabolic syndrome among non-diabetic adults: results from the National Health and Nutrition Examination Survey 1999-2002. *Diabetologia* **2007,** *50* (9), 1841-51.
43. (a) Lim, J. E.; Jee, S. H., Association between serum levels of adiponectin and polychlorinated biphenyls in Korean men and women. *Endocrine* **2014**; (b) Mullerova, D.; Kopecky, J.; Matejkova, D.; Muller, L.; Rosmus, J.; Racek, J.; Sefrna, F.; Opatrna, S.; Kuda, O.; Matejovic, M., Negative association between plasma levels of adiponectin and polychlorinated biphenyl 153 in obese women under non-energy-restrictive regime. *International journal of obesity* **2008,** *32* (12), 1875-8.
44. Mullerova, D.; Kopecky, J., White adipose tissue: storage and effector site for environmental pollutants. *Physiological research / Academia Scientiarum Bohemoslovaca* **2007,** *56* (4), 375-81.
45. Luzardo, O. P.; Henriquez-Hernandez, L. A.; Valeron, P. F.; Lara, P. C.; Almeida-Gonzalez, M.; Losada, A.; Zumbado, M.; Serra-Majem, L.; Alvarez-Leon, E. E.; Boada,

L. D., The relationship between dioxin-like polychlorobiphenyls and IGF-I serum levels in healthy adults: evidence from a cross-sectional study. *PloS one* **2012,** *7* (5), e38213.
46. Organization, W. H., A global brief on hypertension: Silent killer, global public health crisis. *WHO* **2013**.
47. (a) Peters, J. L.; Fabian, M. P.; Levy, J. I., Combined impact of lead, cadmium, polychlorinated biphenyls and non-chemical risk factors on blood pressure in NHANES. *Environmental research* **2014,** *132*, 93-9; (b) Yorita Christensen, K. L.; White, P., A methodological approach to assessing the health impact of environmental chemical mixtures: PCBs and hypertension in the National Health and Nutrition Examination Survey. *International journal of environmental research and public health* **2011,** *8* (11), 4220-37.
48. Ha, M. H.; Lee, D. H.; Son, H. K.; Park, S. K.; Jacobs, D. R., Jr., Association between serum concentrations of persistent organic pollutants and prevalence of newly diagnosed hypertension: results from the National Health and Nutrition Examination Survey 1999-2002. *Journal of human hypertension* **2009,** *23* (4), 274-86.
49. Goncharov, A.; Bloom, M.; Pavuk, M.; Birman, I.; Carpenter, D. O., Blood pressure and hypertension in relation to levels of serum polychlorinated biphenyls in residents of Anniston, Alabama. *J Hypertens* **2010,** *28* (10), 2053-60.
50. Goncharov, A.; Pavuk, M.; Foushee, H. R.; Carpenter, D. O.; Anniston Environmental Health Reseach, C., Blood pressure in relation to concentrations of PCB congeners and chlorinated pesticides. *Environmental health perspectives* **2011,** *119* (3), 319-25.
51. Uemura, H.; Arisawa, K.; Hiyoshi, M.; Kitayama, A.; Takami, H.; Sawachika, F.; Dakeshita, S.; Nii, K.; Satoh, H.; Sumiyoshi, Y.; Morinaga, K.; Kodama, K.; Suzuki, T.; Nagai, M.; Suzuki, T., Prevalence of metabolic syndrome associated with body burden levels of dioxin and related compounds among Japan's general population. *Environmental health perspectives* **2009,** *117* (4), 568-73.
52. Huang, X.; Lessner, L.; Carpenter, D. O., Exposure to persistent organic pollutants and hypertensive disease. *Environmental research* **2006,** *102* (1), 101-6.
53. (a) Akagi, K.; Okumura, M., Association of blood pressure and PCB level in yusho patients. *Environmental health perspectives* **1985,** *59*, 37-9; (b) Stehr-Green, P. A.; Ross, D.; Liddle, J.; Welty, E.; Steele, G., A pilot study of serum polychlorinated biphenyl levels in persons at high risk of exposure in residential and occupational environments. *Archives of environmental health* **1986,** *41* (4), 240-4.
54. (a) Kenchaiah, S.; Evans, J. C.; Levy, D.; Wilson, P. W.; Benjamin, E. J.; Larson, M. G.; Kannel, W. B.; Vasan, R. S., Obesity and the risk of heart failure. *The New England journal of medicine* **2002,** *347* (5), 305-13; (b) Poirier, P.; Giles, T. D.; Bray, G. A.; Hong, Y.; Stern, J. S.; Pi-Sunyer, F. X.; Eckel, R. H.; American Heart, A.; Obesity Committee of the Council on Nutrition, P. A.; Metabolism, Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation* **2006,** *113* (6), 898-918; (c) Van Gaal, L. F.; Mertens, I. L.; De Block, C. E., Mechanisms linking obesity with cardiovascular disease. *Nature* **2006,** *444* (7121), 875-80.
55. (a) Ferrante, M. C.; Amero, P.; Santoro, A.; Monnolo, A.; Simeoli, R.; Di Guida, F.; Mattace Raso, G.; Meli, R., Polychlorinated biphenyls (PCB 101, PCB 153 and PCB 180) alter leptin signaling and lipid metabolism in differentiated 3T3-L1 adipocytes. *Toxicology and applied pharmacology* **2014,** *279* (3), 401-8; (b) Kim, M. J.; Pelloux, V.; Guyot, E.; Tordjman, J.; Bui, L. C.; Chevallier, A.; Forest, C.; Benelli, C.; Clement, K.; Barouki, R., Inflammatory pathway genes belong to major targets of persistent organic

pollutants in adipose cells. *Environmental health perspectives* **2012,** *120* (4), 508-14; (c) Myre, M.; Imbeault, P., Persistent organic pollutants meet adipose tissue hypoxia: does cross-talk contribute to inflammation during obesity? *Obesity reviews : an official journal of the International Association for the Study of Obesity* **2014,** *15* (1), 19-28.
56. (a) Donat-Vargas, C.; Gea, A.; Sayon-Orea, C.; Carlos, S.; Martinez-Gonzalez, M. A.; Bes-Rastrollo, M., Association between dietary intakes of PCBs and the risk of obesity: the SUN project. *J Epidemiol Commun H* **2014,** *68* (9), 834-841; (b) Gladen, B. C.; Ragan, N. B.; Rogan, W. J., Pubertal growth and development and prenatal and lactational exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene. *The Journal of pediatrics* **2000,** *136* (4), 490-6; (c) Lee, D. H.; Lind, L.; Jacobs, D. R., Jr.; Salihovic, S.; van Bavel, B.; Lind, P. M., Associations of persistent organic pollutants with abdominal obesity in the elderly: The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. *Environment international* **2012,** *40*, 170-8; (d) Verhulst, S. L.; Nelen, V.; Hond, E. D.; Koppen, G.; Beunckens, C.; Vael, C.; Schoeters, G.; Desager, K., Intrauterine exposure to environmental pollutants and body mass index during the first 3 years of life. *Environmental health perspectives* **2009,** *117* (1), 122-6.
57. (a) Lignell, S.; Aune, M.; Darnerud, P. O.; Hanberg, A.; Larsson, S. C.; Glynn, A., Prenatal exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) may influence birth weight among infants in a Swedish cohort with background exposure: a cross-sectional study. *Environ Health-Glob* **2013,** *12*; (b) Valvi, D.; Mendez, M. A.; Martinez, D.; Grimalt, J. O.; Torrent, M.; Sunyer, J.; Vrijheid, M., Prenatal Concentrations of Polychlorinated Biphenyls, DDE, and DDT and Overweight in Children: A Prospective Birth Cohort Study. *Environmental health perspectives* **2012,** *120* (3), 451-457.
58. Glynn, A. W.; Granath, F.; Aune, M.; Atuma, S.; Darnerud, P. O.; Bjerselius, R.; Vainio, H.; Weiderpass, E., Organochlorines in Swedish women: Determinants of serum concentrations. *Environmental health perspectives* **2003,** *111* (3), 349-355.
59. (a) Ben Hassine, S.; Hammami, B.; Ben Ameur, W.; El Megdiche, Y.; Barhoumi, B.; El Abidi, R.; Driss, M. R., Concentrations of organochlorine pesticides and polychlorinated biphenyls in human serum and their relation with age, gender, and BMI for the general population of Bizerte, Tunisia. *Environmental science and pollution research international* **2014,** *21* (10), 6303-13; (b) Karmaus, W.; Osuch, J. R.; Eneli, I.; Mudd, L. M.; Zhang, J.; Mikucki, D.; Haan, P.; Davis, S., Maternal levels of dichlorodiphenyl-dichloroethylene (DDE) may increase weight and body mass index in adult female offspring. *Occupational and environmental medicine* **2009,** *66* (3), 143-9.
60. (a) Blanck, H. M.; Marcus, M.; Rubin, C.; Tolbert, P. E.; Hertzberg, V. S.; Henderson, A. K.; Zhang, R. H., Growth in girls exposed in utero and postnatally to polybrominated biphenyls and polychlorinated biphenyls. *Epidemiology* **2002,** *13* (2), 205-10; (b) Dirinck, E. L.; Dirtu, A. C.; Govindan, M.; Covaci, A.; Van Gaal, L. F.; Jorens, P. G., Exposure to persistent organic pollutants: relationship with abnormal glucose metabolism and visceral adiposity. *Diabetes care* **2014,** *37* (7), 1951-8; (c) Dirinck, E.; Jorens, P. G.; Covaci, A.; Geens, T.; Roosens, L.; Neels, H.; Mertens, I.; Van Gaal, L., Obesity and Persistent Organic Pollutants: Possible Obesogenic Effect of Organochlorine Pesticides and Polychlorinated Biphenyls. *Obesity* **2011,** *19* (4), 709-714; (d) Wu, K. S.; Xu, X. J.; Liu, J. X.; Guo, Y. Y.; Huo, X., In utero exposure to polychlorinated biphenyls and reduced neonatal physiological development from Guiyu, China. *Ecotoxicology and environmental safety* **2011,** *74* (8), 2141-2147.
61. (a) Imbeault, P.; Chevrier, J.; Dewailly, E.; Ayotte, P.; Despres, J. P.; Tremblay, A.; Mauriege, P., Increase in plasma pollutant levels in response to weight loss in humans is related to in vitro subcutaneous adipocyte basal lipolysis. *International journal of obesity and related metabolic disorders : journal of the International Association for*

the Study of Obesity **2001,** *25* (11), 1585-91; (b) Yu, G. W.; Laseter, J.; Mylander, C., Persistent organic pollutants in serum and several different fat compartments in humans. *Journal of environmental and public health* **2011,** *2011*, 417980.
62. (a) Baillie-Hamilton, P. F., Chemical toxins: a hypothesis to explain the global obesity epidemic. *Journal of alternative and complementary medicine* **2002,** *8* (2), 185-92; (b) Grun, F.; Blumberg, B., Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* **2006,** *147* (6 Suppl), S50-5.
63. (a) Huffman, M. D.; Lloyd-Jones, D. M.; Ning, H.; Labarthe, D. R.; Guzman Castillo, M.; O'Flaherty, M.; Ford, E. S.; Capewell, S., Quantifying options for reducing coronary heart disease mortality by 2020. *Circulation* **2013,** *127* (25), 2477-84; (b) Martin, S. S.; Blaha, M. J.; Blankstein, R.; Agatston, A.; Rivera, J. J.; Virani, S. S.; Ouyang, P.; Jones, S. R.; Blumenthal, R. S.; Budoff, M. J.; Nasir, K., Dyslipidemia, coronary artery calcium, and incident atherosclerotic cardiovascular disease: implications for statin therapy from the multi-ethnic study of atherosclerosis. *Circulation* **2014,** *129* (1), 77-86; (c) Riche, D. M.; McClendon, K. S., Role of statins for the primary prevention of cardiovascular disease in patients with type 2 diabetes mellitus. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* **2007,** *64* (15), 1603-10.
64. Tenenbaum, A.; Klempfner, R.; Fisman, E. Z., Hypertriglyceridemia: a too long unfairly neglected major cardiovascular risk factor. *Cardiovasc Diabetol* **2014,** *13* (1), 159.
65. (a) Bell, F. P.; Iverson, F.; Arnold, D.; Vidmar, T. J., Long-term effects of Aroclor 1254 (PCBs) on plasma lipid and carnitine concentrations in rhesus monkey. *Toxicology* **1994,** *89* (2), 139-53; (b) Nagaoka, S.; Miyazaki, H.; Aoyama, Y.; Yoshida, A., Effects of dietary polychlorinated biphenyls on cholesterol catabolism in rats. *The British journal of nutrition* **1990,** *64* (1), 161-9; (c) Nagaoka, S.; Kato, M.; Aoyama, Y.; Yoshida, A., Comparative studies on the hypercholesterolaemia induced by excess dietary tyrosine or polychlorinated biphenyls in rats. *The British journal of nutrition* **1986,** *56* (2), 509-17.
66. (a) Baker, E. L., Jr.; Landrigan, P. J.; Glueck, C. J.; Zack, M. M., Jr.; Liddle, J. A.; Burse, V. W.; Housworth, W. J.; Needham, L. L., Metabolic consequences of exposure to polychlorinated biphenyls (PCB) in sewage sludge. *Am J Epidemiol* **1980,** *112* (4), 553-63; (b) Chase, K. H.; Wong, O.; Thomas, D.; Berney, B. W.; Simon, R. K., Clinical and metabolic abnormalities associated with occupational exposure to polychlorinated biphenyls (PCBs). *Journal of occupational medicine. : official publication of the Industrial Medical Association* **1982,** *24* (2), 109-14; (c) Smith, A. B.; Schloemer, J.; Lowry, L. K.; Smallwood, A. W.; Ligo, R. N.; Tanaka, S.; Stringer, W.; Jones, M.; Hervin, R.; Glueck, C. J., Metabolic and health consequences of occupational exposure to polychlorinated biphenyls. *British journal of industrial medicine* **1982,** *39* (4), 361-9; (d) Stehrgreen, P. A.; Welty, E.; Steele, G.; Steinberg, K., Evaluation of Potential Health-Effects Associated with Serum Polychlorinated Biphenyl Levels. *Environmental health perspectives* **1986,** *70*, 255-259.
67. (a) Aminov, Z.; Haase, R. F.; Pavuk, M.; Carpenter, D. O.; Anniston Environmental Health Research, C., Analysis of the effects of exposure to polychlorinated biphenyls and chlorinated pesticides on serum lipid levels in residents of Anniston, Alabama. *Environmental health : a global access science source* **2013,** *12*, 108; (b) Penell, J.; Lind, L.; Salihovic, S.; van Bavel, B.; Lind, P. M., Persistent organic pollutants are related to the change in circulating lipid levels during a 5 year follow-up. *Environmental research* **2014,** *134*, 190-7.
68. Tokunaga, S.; Kataoka, K., [A longitudinal analysis on the association of serum lipids and lipoproteins concentrations with blood polychlorinated biphenyls level in

chronic "Yusho" patients]. *Fukuoka igaku zasshi = Hukuoka acta medica* **2003,** *94* (5), 110-7.
69. Linsel-Nitschke, P.; Tall, A. R., HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nature reviews. Drug discovery* **2005,** *4* (3), 193-205.
70. Beischlag, T. V.; Luis Morales, J.; Hollingshead, B. D.; Perdew, G. H., The aryl hydrocarbon receptor complex and the control of gene expression. *Critical reviews in eukaryotic gene expression* **2008,** *18* (3), 207-50.
71. Cox, M. B.; Miller, C. A., 3rd, Cooperation of heat shock protein 90 and p23 in aryl hydrocarbon receptor signaling. *Cell stress & chaperones* **2004,** *9* (1), 4-20.
72. Korashy, H. M.; El-Kadi, A. O., The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases. *Drug metabolism reviews* **2006,** *38* (3), 411-50.
73. (a) Xiao, L.; Zhang, Z.; Luo, X., Roles of xenobiotic receptors in vascular pathophysiology. *Circ J* **2014,** *78* (7), 1520-30; (b) Han, S. G.; Han, S. S.; Toborek, M.; Hennig, B., EGCG protects endothelial cells against PCB 126-induced inflammation through inhibition of AhR and induction of Nrf2-regulated genes. *Toxicology and applied pharmacology* **2012,** *261* (2), 181-8.
74. Guengerich, F. P., Cytochrome p450 and chemical toxicology. *Chemical research in toxicology* **2008,** *21* (1), 70-83.
75. (a) Ceriello, A.; Motz, E., Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arteriosclerosis, thrombosis, and vascular biology* **2004,** *24* (5), 816-23; (b) Marseglia, L.; Manti, S.; D'Angelo, G.; Nicotera, A.; Parisi, E.; Di Rosa, G.; Gitto, E.; Arrigo, T., Oxidative Stress in Obesity: A Critical Component in Human Diseases. *International journal of molecular sciences* **2014,** *16* (1), 378-400; (c) Ward, N. C.; Croft, K. D., Hypertension and oxidative stress. *Clinical and experimental pharmacology & physiology* **2006,** *33* (9), 872-6.
76. Dhalla, N. S.; Temsah, R. M.; Netticadan, T., Role of oxidative stress in cardiovascular diseases. *J Hypertens* **2000,** *18* (6), 655-73.
77. Cai, H.; Harrison, D. G., Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circulation research* **2000,** *87* (10), 840-4.
78. Heitzer, T.; Schlinzig, T.; Krohn, K.; Meinertz, T.; Munzel, T., Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation* **2001,** *104* (22), 2673-8.
79. Wu, F.; Zheng, Y.; Gao, J.; Chen, S.; Wang, Z., Induction of oxidative stress and the transcription of genes related to apoptosis in rare minnow (Gobiocypris rarus) larvae with Aroclor 1254 exposure. *Ecotoxicology and environmental safety* **2014,** *110*, 254-60.
80. Baker, R. G.; Hayden, M. S.; Ghosh, S., NF-kappaB, inflammation, and metabolic disease. *Cell metabolism* **2011,** *13* (1), 11-22.
81. (a) Collins, T.; Cybulsky, M. I., NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? *J Clin Invest* **2001,** *107* (3), 255-64; (b) Mangge, H.; Becker, K.; Fuchs, D.; Gostner, J. M., Antioxidants, inflammation and cardiovascular disease. *World journal of cardiology* **2014,** *6* (6), 462-77.
82. (a) Baird, L.; Dinkova-Kostova, A. T., The cytoprotective role of the Keap1-Nrf2 pathway. *Archives of toxicology* **2011,** *85* (4), 241-72; (b) Kansanen, E.; Jyrkkanen, H. K.; Levonen, A. L., Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free radical biology & medicine* **2012,** *52* (6), 973-82.
83. (a) Kopf, P. G.; Huwe, J. K.; Walker, M. K., Hypertension, cardiac hypertrophy, and impaired vascular relaxation induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin are associated with increased superoxide. *Cardiovascular toxicology* **2008,** *8* (4), 181-93; (b) Kopf, P. G.; Scott, J. A.; Agbor, L. N.; Boberg, J. R.; Elased, K. M.; Huwe, J. K.; Walker,

M. K., Cytochrome P4501A1 is required for vascular dysfunction and hypertension induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicological sciences : an official journal of the Society of Toxicology* **2010,** *117* (2), 537-46.
84. (a) Zhang, N., The role of endogenous aryl hydrocarbon receptor signaling in cardiovascular physiology. *Journal of cardiovascular disease research* **2011,** *2* (2), 91-5; (b) Nguyen, L. P.; Bradfield, C. A., The search for endogenous activators of the aryl hydrocarbon receptor. *Chemical research in toxicology* **2008,** *21* (1), 102-16.
85. Huang, S.; Shui, X.; He, Y.; Xue, Y.; Li, J.; Li, G.; Lei, W.; Chen, C., AhR expression and polymorphisms are associated with risk of coronary arterial disease in Chinese population. *Scientific reports* **2015,** *5*, 8022.
86. (a) Pascussi, J. M.; Gerbal-Chaloin, S.; Duret, C.; Daujat-Chavanieu, M.; Vilarem, M. J.; Maurel, P., The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. *Annu Rev Pharmacol Toxicol* **2008,** *48*, 1-32; (b) Wakabayashi, N.; Slocum, S. L.; Skoko, J. J.; Shin, S.; Kensler, T. W., When NRF2 talks, who's listening? *Antioxidants & redox signaling* **2010,** *13* (11), 1649-63.
87. (a) Tian, Y., Ah receptor and NF-kappaB interplay on the stage of epigenome. *Biochemical pharmacology* **2009,** *77* (4), 670-80; (b) Zordoky, B. N.; El-Kadi, A. O., Role of NF-kappaB in the regulation of cytochrome P450 enzymes. *Current drug metabolism* **2009,** *10* (2), 164-78.
88. Frank, P. G.; Woodman, S. E.; Park, D. S.; Lisanti, M. P., Caveolin, caveolae, and endothelial cell function. *Arteriosclerosis, thrombosis, and vascular biology* **2003,** *23* (7), 1161-8.
89. Smart, E. J.; Graf, G. A.; McNiven, M. A.; Sessa, W. C.; Engelman, J. A.; Scherer, P. E.; Okamoto, T.; Lisanti, M. P., Caveolins, liquid-ordered domains, and signal transduction. *Molecular and cellular biology* **1999,** *19* (11), 7289-304.
90. Sowa, G., Caveolae, caveolins, cavins, and endothelial cell function: new insights. *Frontiers in physiology* **2012,** *2*, 120.
91. Trigatti, B. L.; Anderson, R. G.; Gerber, G. E., Identification of caveolin-1 as a fatty acid binding protein. *Biochemical and biophysical research communications* **1999,** *255* (1), 34-9.
92. Panneerselvam, M.; Patel, H. H.; Roth, D. M., Caveolins and heart diseases. *Advances in experimental medicine and biology* **2012,** *729*, 145-56.
93. Pavlides, S.; Gutierrez-Pajares, J. L.; Danilo, C.; Lisanti, M. P.; Frank, P. G., Atherosclerosis, caveolae and caveolin-1. *Advances in experimental medicine and biology* **2012,** *729*, 127-44.
94. Frank, P. G.; Lee, H.; Park, D. S.; Tandon, N. N.; Scherer, P. E.; Lisanti, M. P., Genetic ablation of caveolin-1 confers protection against atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **2004,** *24* (1), 98-105.
95. Fernandez-Hernando, C.; Yu, J.; Davalos, A.; Prendergast, J.; Sessa, W. C., Endothelial-specific overexpression of caveolin-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *The American journal of pathology* **2010,** *177* (2), 998-1003.
96. Frank, P. G., Endothelial caveolae and caveolin-1 as key regulators of atherosclerosis. *The American journal of pathology* **2010,** *177* (2), 544-6.
97. Majkova, Z.; Toborek, M.; Hennig, B., The role of caveolae in endothelial cell dysfunction with a focus on nutrition and environmental toxicants. *J Cell Mol Med* **2010,** *14* (10), 2359-70.
98. Petriello, M. C.; Han, S. G.; Newsome, B. J.; Hennig, B., PCB 126 toxicity is modulated by cross-talk between caveolae and Nrf2 signaling. *Toxicology and applied pharmacology* **2014,** *277* (2), 192-9.

99. (a) Niture, S. K.; Khatri, R.; Jaiswal, A. K., Regulation of Nrf2-an update. *Free radical biology & medicine* **2014,** *66*, 36-44; (b) Niture, S. K.; Jain, A. K.; Jaiswal, A. K., Antioxidant-induced modification of INrf2 cysteine 151 and PKC-delta-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. *Journal of cell science* **2009,** *122* (Pt 24), 4452-64.
100. Kim, J.; Cha, Y. N.; Surh, Y. J., A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutation research* **2010,** *690* (1-2), 12-23.
101. Turpaev, K. T., Keap1-Nrf2 signaling pathway: mechanisms of regulation and role in protection of cells against toxicity caused by xenobiotics and electrophiles. *Biochemistry. Biokhimiia* **2013,** *78* (2), 111-26.
102. Singh, S.; Vrishni, S.; Singh, B. K.; Rahman, I.; Kakkar, P., Nrf2-ARE stress response mechanism: a control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases. *Free Radic Res 44* (11), 1267-88.
103. Kim, J.; Cha, Y. N.; Surh, Y. J., A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res 690* (1-2), 12-23.
104. Howden, R., Nrf2 and cardiovascular defense. *Oxidative medicine and cellular longevity* **2013,** *2013*, 104308.
105. Takabe, W.; Warabi, E.; Noguchi, N., Anti-atherogenic effect of laminar shear stress via Nrf2 activation. *Antioxidants & redox signaling* **2011,** *15* (5), 1415-26.
106. Hosoya, T.; Maruyama, A.; Kang, M. I.; Kawatani, Y.; Shibata, T.; Uchida, K.; Warabi, E.; Noguchi, N.; Itoh, K.; Yamamoto, M., Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells. *The Journal of biological chemistry* **2005,** *280* (29), 27244-50.
107. Lu, H.; Cui, W.; Klaassen, C. D., Nrf2 protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced oxidative injury and steatohepatitis. *Toxicology and applied pharmacology* **2011,** *256* (2), 122-35.
108. (a) Ganguly, R.; Pierce, G. N., Trans fat involvement in cardiovascular disease. *Molecular nutrition & food research* **2012,** *56* (7), 1090-6; (b) Baum, S. J.; Kris-Etherton, P. M.; Willett, W. C.; Lichtenstein, A. H.; Rudel, L. L.; Maki, K. C.; Whelan, J.; Ramsden, C. E.; Block, R. C., Fatty acids in cardiovascular health and disease: a comprehensive update. *Journal of clinical lipidology* **2012,** *6* (3), 216-34; (c) Kuipers, R. S.; de Graaf, D. J.; Luxwolda, M. F.; Muskiet, M. H.; Dijck-Brouwer, D. A.; Muskiet, F. A., Saturated fat, carbohydrates and cardiovascular disease. *The Netherlands journal of medicine* **2011,** *69* (9), 372-8.
109. Hennig, B.; Slim, R.; Toborek, M.; Robertson, L. W., Linoleic acid amplifies polychlorinated biphenyl-mediated dysfunction of endothelial cells. *Journal of biochemical and molecular toxicology* **1999,** *13* (2), 83-91.
110. Wu, J.; Liu, J.; Waalkes, M. P.; Cheng, M. L.; Li, L.; Li, C. X.; Yang, Q., High dietary fat exacerbates arsenic-induced liver fibrosis in mice. *Experimental biology and medicine (Maywood, N.J.)* **2008,** *233* (3), 377-84.
111. Harris, D. L.; Niaz, M. S.; Morrow, J. D.; Mary, K.; Ramesh, A., Diet as a Modifier of Benzo (a) pyrene Metabolism and Benzo (a) pyrene—Induced Colon Tumors in ApcMin mice. *Interdisciplinary Studies on Environmental Chemistry—Environmental Research in Asia* **2009,** *2*, 227-238.
112. Hennig, B.; Reiterer, G.; Toborek, M.; Matveev, S. V.; Daugherty, A.; Smart, E.; Robertson, L. W., Dietary fat interacts with PCBs to induce changes in lipid metabolism in mice deficient in low-density lipoprotein receptor. *Environmental health perspectives* **2005,** *113* (1), 83-7.

113. NCI, Table 2. Food sources of the total omega 6 fatty acids (18:2 + 20:4), listed in descending order by percentages of their contribution to intake, based on data from the national health and nutrition examination survey 2005–2006. *NIH* **2013**.
114. (a) Fresno, M.; Alvarez, R.; Cuesta, N., Toll-like receptors, inflammation, metabolism and obesity. *Archives of physiology and biochemistry* **2011,** *117* (3), 151-64; (b) Nebert, D. W.; Karp, C. L., Endogenous functions of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology. *The Journal of biological chemistry* **2008,** *283* (52), 36061-5; (c) Tompkins, L. M.; Wallace, A. D., Mechanisms of cytochrome P450 induction. *Journal of biochemical and molecular toxicology* **2007,** *21* (4), 176-81.
115. (a) Slim, R.; Toborek, M.; Robertson, L. W.; Hennig, B., Antioxidant protection against PCB-mediated endothelial cell activation. *Toxicological sciences : an official journal of the Society of Toxicology* **1999,** *52* (2), 232-9; (b) Watkins, B. A.; Hannon, K.; Ferruzzi, M.; Li, Y., Dietary PUFA and flavonoids as deterrents for environmental pollutants. *The Journal of nutritional biochemistry* **2007,** *18* (3), 196-205.
116. (a) Majkova, Z.; Layne, J.; Sunkara, M.; Morris, A. J.; Toborek, M.; Hennig, B., Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls. *Toxicol Appl Pharmacol* **2011,** *251* (1), 41-9; (b) Zheng, Y.; Morris, A.; Sunkara, M.; Layne, J.; Toborek, M.; Hennig, B., Epigallocatechin-gallate stimulates NF-E2-related factor and heme oxygenase-1 via caveolin-1 displacement. *The Journal of nutritional biochemistry* **2012,** *23* (2), 163-8.
117. Terao, J., Dietary flavonoids as antioxidants. *Forum of nutrition* **2009,** *61*, 87-94.
118. Morita, K.; Matsueda, T.; Iida, T., Effect of green tea (matcha) on gastrointestinal tract absorption of polychlorinated biphenyls, polychlorinated dibenzofurans and polychlorinated dibenzo-p-dioxins in rats. *Fukuoka igaku zasshi = Hukuoka acta medica* **1997,** *88* (5), 162-8.
119. Ciftci, O.; Ozdemir, I., Protective effects of quercetin and chrysin against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced oxidative stress, body wasting and altered cytokine productions in rats. *Immunopharmacology and immunotoxicology* **2011,** *33* (3), 504-8.
120. Zheng, Y.; Toborek, M.; Hennig, B., Epigallocatechin gallate-mediated protection against tumor necrosis factor-alpha-induced monocyte chemoattractant protein-1 expression is heme oxygenase-1 dependent. *Metabolism: clinical and experimental* **2010,** *59* (10), 1528-35.
121. Tutel'yan, V. A.; Gapparov, M. M.; Telegin, L. Y.; Devichenskii, V. M.; Pevnitskii, L. A., Flavonoids and resveratrol as regulators of Ah-receptor activity: protection from dioxin toxicity. *Bulletin of experimental biology and medicine* **2003,** *136* (6), 533-9.
122. Wu, J. M.; Wang, Z. R.; Hsieh, T. C.; Bruder, J. L.; Zou, J. G.; Huang, Y. Z., Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review). *International journal of molecular medicine* **2001,** *8* (1), 3-17.
123. (a) Baur, J. A.; Sinclair, D. A., Therapeutic potential of resveratrol: the in vivo evidence. *Nature reviews. Drug discovery* **2006,** *5* (6), 493-506; (b) Higgins, L. G.; Hayes, J. D., Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. *Drug metabolism reviews* **2011,** *43* (2), 92-137; (c) Kang, H. J.; Hong, Y. B.; Kim, H. J.; Wang, A.; Bae, I., Bioactive food components prevent carcinogenic stress via Nrf2 activation in BRCA1 deficient breast epithelial cells. *Toxicology letters* **2012,** *209* (2), 154-60; (d) Nadtochiy, S. M.; Redman, E. K., Mediterranean diet and cardioprotection: the role of nitrite, polyunsaturated fatty acids, and polyphenols. *Nutrition (Burbank, Los Angeles County, Calif.)* **2011,** *27* (7-8), 733-44; (e) Zakkar, M.; Van der Heiden, K.; Luong le, A.; Chaudhury, H.; Cuhlmann, S.; Hamdulay, S. S.; Krams, R.; Edirisinghe, I.; Rahman, I.;

Carlsen, H.; Haskard, D. O.; Mason, J. C.; Evans, P. C., Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state. *Arteriosclerosis, thrombosis, and vascular biology* **2009,** *29* (11), 1851-7.
124. (a) Turkez, H.; Geyikoglu, F.; Yousef, M. I., Ameliorative effect of docosahexaenoic acid on 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced histological changes, oxidative stress, and DNA damage in rat liver. *Toxicology and industrial health* **2012,** *28* (8), 687-96; (b) Mozaffarian, D.; Wu, J. H., Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *Journal of the American College of Cardiology* **2011,** *58* (20), 2047-67.
125. (a) Watkins, B. A.; Li, Y.; Seifert, M. F., Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. *The Journal of nutritional biochemistry* **2006,** *17* (4), 282-9; (b) Gomez Candela, C.; Bermejo Lopez, L. M.; Loria Kohen, V., Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: nutritional recommendations. *Nutricion hospitalaria : organo oficial de la Sociedad Espanola de Nutricion Parenteral y Enteral* **2011,** *26* (2), 323-9.
126. Wang, L.; Reiterer, G.; Toborek, M.; Hennig, B., Changing ratios of omega-6 to omega-3 fatty acids can differentially modulate polychlorinated biphenyl toxicity in endothelial cells. *Chemico-biological interactions* **2008,** *172* (1), 27-38.
127. Eske, K. Coplanar PCB-induced inflammation and dietary interventions. University of Kentucky, UKnowledge, 2013.
128. (a) Choi, Y. J.; Arzuaga, X.; Kluemper, C. T.; Caraballo, A.; Toborek, M.; Hennig, B., Quercetin blocks caveolae-dependent pro-inflammatory responses induced by co-planar PCBs. *Environment international* **2010,** *36* (8), 931-4; (b) Ciftci, O.; Aydin, M.; Ozdemir, I.; Vardi, N., Quercetin prevents 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced testicular damage in rats. *Andrologia* **2012,** *44* (3), 164-73; (c) Sciullo, E. M.; Vogel, C. F.; Wu, D.; Murakami, A.; Ohigashi, H.; Matsumura, F., Effects of selected food phytochemicals in reducing the toxic actions of TCDD and p,p'-DDT in U937 macrophages. *Archives of toxicology* **2010,** *84* (12), 957-66.
129. (a) Morita, K.; Matsueda, T.; Iida, T., [Effect of green tea (matcha) on gastrointestinal tract absorption of polychlorinated biphenyls, polychlorinated dibenzofurans and polychlorinated dibenzo-p-dioxins in rats]. *Fukuoka igaku zasshi = Hukuoka acta medica* **1997,** *88* (5), 162-8; (b) Newsome, B. J.; Petriello, M. C.; Han, S. G.; Murphy, M. O.; Eske, K. E.; Sunkara, M.; Morris, A. J.; Hennig, B., Green tea diet decreases PCB 126-induced oxidative stress in mice by up-regulating antioxidant enzymes. *The Journal of nutritional biochemistry* **2014,** *25* (2), 126-35.
130. (a) Kim, J.; Koo, S. I.; Noh, S. K., Green tea extract markedly lowers the lymphatic absorption and increases the biliary secretion of 14C-benzo[a]pyrene in rats. *The Journal of nutritional biochemistry* **2012,** *23* (8), 1007-11; (b) Koo, S. I.; Noh, S. K., Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. *The Journal of nutritional biochemistry* **2007,** *18* (3), 179-83.
131. Yaktine, A. L.; Harrison, G. G.; Lawrence, R. S., Reducing exposure to dioxins and related compounds through foods in the next generation. *Nutrition reviews* **2006,** *64* (9), 403-9.
132. (a) Aozasa, O.; Ohta, S.; Nakao, T.; Miyata, H.; Nomura, T., Enhancement in fecal excretion of dioxin isomer in mice by several dietary fibers. *Chemosphere* **2001,** *45* (2), 195-200; (b) De Vos, S.; De Schrijver, R., Polychlorinated biphenyl distribution and faecal excretion in rats fed wheat bran. *Chemosphere* **2005,** *61* (3), 374-82; (c) Sera, N.; Morita, K.; Nagasoe, M.; Tokieda, H.; Kitaura, T.; Tokiwa, H., Binding effect of polychlorinated compounds and environmental carcinogens on rice bran fiber. *The Journal of nutritional biochemistry* **2005,** *16* (1), 50-8.

133. (a) Arguin, H.; Sanchez, M.; Bray, G. A.; Lovejoy, J. C.; Peters, J. C.; Jandacek, R. J.; Chaput, J. P.; Tremblay, A., Impact of adopting a vegan diet or an olestra supplementation on plasma organochlorine concentrations: results from two pilot studies. *The British journal of nutrition* **2010,** *103* (10), 1433-41; (b) Geusau, A.; Tschachler, E.; Meixner, M.; Sandermann, S.; Papke, O.; Wolf, C.; Valic, E.; Stingl, G.; McLachlan, M., Olestra increases faecal excretion of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Lancet* **1999,** *354* (9186), 1266-7; (c) Geusau, A.; Schmaldienst, S.; Derfler, K.; Papke, O.; Abraham, K., Severe 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD) intoxication: kinetics and trials to enhance elimination in two patients. *Archives of toxicology* **2002,** *76* (5-6), 316-25; (d) Jandacek, R. J.; Rider, T.; Keller, E. R.; Tso, P., The effect of olestra on the absorption, excretion and storage of 2,2',5,5' tetrachlorobiphenyl; 3,3',4,4' tetrachlorobiphenyl; and perfluorooctanoic acid. *Environment international* **2010,** *36* (8), 880-3.
134. Moser, G. A.; McLachlan, M. S., A non-absorbable dietary fat substitute enhances elimination of persistent lipophilic contaminants in humans. *Chemosphere* **1999,** *39* (9), 1513-21.
135. Redgrave, T. G.; Wallace, P.; Jandacek, R. J.; Tso, P., Treatment with a dietary fat substitute decreased Arochlor 1254 contamination in an obese diabetic male. *The Journal of nutritional biochemistry* **2005,** *16* (6), 383-4.
136. (a) Singh, S.; Vrishni, S.; Singh, B. K.; Rahman, I.; Kakkar, P., Nrf2-ARE stress response mechanism: a control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases. *Free radical research* **2010,** *44* (11), 1267-88; (b) Itoh, K.; Mimura, J.; Yamamoto, M., Discovery of the negative regulator of Nrf2, Keap1: a historical overview. *Antioxidants & redox signaling* **2010,** *13* (11), 1665-78.
137. (a) Miao, X.; Bai, Y.; Sun, W.; Cui, W.; Xin, Y.; Wang, Y.; Tan, Y.; Miao, L.; Fu, Y.; Su, G.; Cai, L., Sulforaphane prevention of diabetes-induced aortic damage was associated with the up-regulation of Nrf2 and its down-stream antioxidants. *Nutrition & metabolism* **2012,** *9* (1), 84; (b) Nair, S.; Barve, A.; Khor, T. O.; Shen, G. X.; Lin, W.; Chan, J. Y.; Cai, L.; Kong, A. N., Regulation of Nrf2- and AP-1-mediated gene expression by epigallocatechin-3-gallate and sulforaphane in prostate of Nrf2-knockout or C57BL/6J mice and PC-3 AP-1 human prostate cancer cells. *Acta pharmacologica Sinica* **2010,** *31* (9), 1223-40.
138. Hayes, J. D.; Dinkova-Kostova, A. T.; McMahon, M., Cross-talk between transcription factors AhR and Nrf2: lessons for cancer chemoprevention from dioxin. *Toxicological sciences : an official journal of the Society of Toxicology* **2009,** *111* (2), 199-201.
139. Yeager, R. L.; Reisman, S. A.; Aleksunes, L. M.; Klaassen, C. D., Introducing the "TCDD-inducible AhR-Nrf2 gene battery". *Toxicological sciences : an official journal of the Society of Toxicology* **2009,** *111* (2), 238-46.
140. Li, W.; Liu, H.; Zhou, J. S.; Cao, J. F.; Zhou, X. B.; Choi, A. M.; Chen, Z. H.; Shen, H. H., Caveolin-1 inhibits expression of antioxidant enzymes through direct interaction with nuclear erythroid 2 p45-related factor-2 (Nrf2). *The Journal of biological chemistry* **2012,** *287* (25), 20922-30.
141. (a) Wang, L. X.; Heredia, A.; Song, H.; Zhang, Z.; Yu, B.; Davis, C.; Redfield, R., Resveratrol glucuronides as the metabolites of resveratrol in humans: characterization, synthesis, and anti-HIV activity. *Journal of pharmaceutical sciences* **2004,** *93* (10), 2448-57; (b) Urpi-Sarda, M.; Zamora-Ros, R.; Lamuela-Raventos, R.; Cherubini, A.; Jauregui, O.; de la Torre, R.; Covas, M. I.; Estruch, R.; Jaeger, W.; Andres-Lacueva, C., HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clinical chemistry* **2007,** *53* (2), 292-9.

142. Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; Rice-Evans, C. A., The metabolic fate of dietary polyphenols in humans. *Free radical biology & medicine* **2002,** *33* (2), 220-35.
143. (a) Fam, S. S.; Murphey, L. J.; Terry, E. S.; Zackert, W. E.; Chen, Y.; Gao, L.; Pandalai, S.; Milne, G. L.; Roberts, L. J.; Porter, N. A.; Montine, T. J.; Morrow, J. D., Formation of highly reactive A-ring and J-ring isoprostane-like compounds (A4/J4-neuroprostanes) in vivo from docosahexaenoic acid. *The Journal of biological chemistry* **2002,** *277* (39), 36076-84; (b) Gao, L.; Wang, J.; Sekhar, K. R.; Yin, H.; Yared, N. F.; Schneider, S. N.; Sasi, S.; Dalton, T. P.; Anderson, M. E.; Chan, J. Y.; Morrow, J. D.; Freeman, M. L., Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. *The Journal of biological chemistry* **2007,** *282* (4), 2529-37; (c) Musiek, E. S.; Brooks, J. D.; Joo, M.; Brunoldi, E.; Porta, A.; Zanoni, G.; Vidari, G.; Blackwell, T. S.; Montine, T. J.; Milne, G. L.; McLaughlin, B.; Morrow, J. D., Electrophilic cyclopentenone neuroprostanes are anti-inflammatory mediators formed from the peroxidation of the omega-3 polyunsaturated fatty acid docosahexaenoic acid. *The Journal of biological chemistry* **2008,** *283* (29), 19927-35.
144. (a) Khoo, N. K.; Rudolph, V.; Cole, M. P.; Golin-Bisello, F.; Schopfer, F. J.; Woodcock, S. R.; Batthyany, C.; Freeman, B. A., Activation of vascular endothelial nitric oxide synthase and heme oxygenase-1 expression by electrophilic nitro-fatty acids. *Free radical biology & medicine* **2010,** *48* (2), 230-9; (b) Khoo, N. K.; Freeman, B. A., Electrophilic nitro-fatty acids: anti-inflammatory mediators in the vascular compartment. *Current opinion in pharmacology* **2010,** *10* (2), 179-84; (c) Rudolph, V.; Rudolph, T. K.; Schopfer, F. J.; Bonacci, G.; Woodcock, S. R.; Cole, M. P.; Baker, P. R.; Ramani, R.; Freeman, B. A., Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovascular research* **2010,** *85* (1), 155-66; (d) Rudolph, T. K.; Rudolph, V.; Edreira, M. M.; Cole, M. P.; Bonacci, G.; Schopfer, F. J.; Woodcock, S. R.; Franek, A.; Pekarova, M.; Khoo, N. K.; Hasty, A. H.; Baldus, S.; Freeman, B. A., Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice. *Arteriosclerosis, thrombosis, and vascular biology* **2010,** *30* (5), 938-45; (e) Tsujita, T.; Li, L.; Nakajima, H.; Iwamoto, N.; Nakajima-Takagi, Y.; Ohashi, K.; Kawakami, K.; Kumagai, Y.; Freeman, B. A.; Yamamoto, M.; Kobayashi, M., Nitro-fatty acids and cyclopentenone prostaglandins share strategies to activate the Keap1-Nrf2 system: a study using green fluorescent protein transgenic zebrafish. *Genes to cells : devoted to molecular & cellular mechanisms* **2011,** *16* (1), 46-57.
145. Freeman, B. A.; Baker, P. R.; Schopfer, F. J.; Woodcock, S. R.; Napolitano, A.; d'Ischia, M., Nitro-fatty acid formation and signaling. *The Journal of biological chemistry* **2008,** *283* (23), 15515-9.
146. Baker, P. R.; Schopfer, F. J.; O'Donnell, V. B.; Freeman, B. A., Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids. *Free radical biology & medicine* **2009,** *46* (8), 989-1003.
147. (a) Kansanen, E.; Bonacci, G.; Schopfer, F. J.; Kuosmanen, S. M.; Tong, K. I.; Leinonen, H.; Woodcock, S. R.; Yamamoto, M.; Carlberg, C.; Yla-Herttuala, S.; Freeman, B. A.; Levonen, A. L., Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism. *The Journal of biological chemistry* **2011,** *286* (16), 14019-27; (b) Borniquel, S.; Jansson, E. A.; Cole, M. P.; Freeman, B. A.; Lundberg, J. O., Nitrated oleic acid up-regulates PPARgamma and attenuates experimental inflammatory bowel disease. *Free radical biology & medicine* **2010,** *48* (4), 499-505.
148. Goncharov, A.; Haase, R. F.; Santiago-Rivera, A.; Morse, G.; Akwesasne Task Force on the, E.; McCaffrey, R. J.; Rej, R.; Carpenter, D. O., High serum PCBs are associated with elevation of serum lipids and cardiovascular disease in a Native American population. *Environmental research* **2008,** *106* (2), 226-39.

149. (a) ATSDR, Polychlorinated Biphenyls (PCB) Toxicity

Exposure Pathways. HEALTH, D. O.; SERVICES, A. H., Eds. http://www.atsdr.cdc.gov/csem/csem.asp?csem=22&po=4, 2000; (b) Faroon, O.; Samuel Keith, L.; Smith-Simon, C.; De Rosa, C., POLYCHLORINATED BIPHENYLS:HUMAN HEALTH ASPECTS. Organization, W. H., Ed. Geneva, 2003; pp 1-64.
150. Libby, P., What have we learned about the biology of atherosclerosis? The role of inflammation. *The American journal of cardiology* **2001,** *88* (7B), 3J-6J.
151. Layne, J.; Majkova, Z.; Smart, E. J.; Toborek, M.; Hennig, B., Caveolae: a regulatory platform for nutritional modulation of inflammatory diseases. *The Journal of nutritional biochemistry* **2011,** *22* (9), 807-11.
152. Croce, K.; Libby, P., Intertwining of thrombosis and inflammation in atherosclerosis. *Current opinion in hematology* **2007,** *14* (1), 55-61.
153. Niture, S. K.; Khatri, R.; Jaiswal, A. K., Regulation of Nrf2-an update. *Free radical biology & medicine* **2013**.
154. Wang, L.; Chen, Y.; Sternberg, P.; Cai, J., Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Investigative ophthalmology & visual science* **2008,** *49* (4), 1671-8.
155. (a) Newsome, B.; Petriello, M.; Han, S.; Murphy, M.; Eske, K.; Sunkara, M.; Morris, A.; Hennig, B., Green tea diet decreases PCB 126-induced oxidative stress in mice by upregulating antioxidant

enzymes. *Journal of nutritional biochemistry* **2013**; (b) Park, S. H.; Jang, J. H.; Chen, C. Y.; Na, H. K.; Surh, Y. J., A formulated red ginseng extract rescues PC12 cells from PCB-induced oxidative cell death through Nrf2-mediated upregulation of heme oxygenase-1 and glutamate cysteine ligase. *Toxicology* **2010,** *278* (1), 131-9.
156. (a) Bellezza, I.; Tucci, A.; Galli, F.; Grottelli, S.; Mierla, A. L.; Pilolli, F.; Minelli, A., Inhibition of NF-kappaB nuclear translocation via HO-1 activation underlies alpha-tocopheryl succinate toxicity. *The Journal of nutritional biochemistry* **2012,** *23* (12), 1583-91; (b) Yang, Y. C.; Lii, C. K.; Wei, Y. L.; Li, C. C.; Lu, C. Y.; Liu, K. L.; Chen, H. W., Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-kappaB pathways. *The Journal of nutritional biochemistry* **2013,** *24* (1), 204-12.
157. Salous, A. K.; Panchatcharam, M.; Sunkara, M.; Mueller, P.; Dong, A.; Wang, Y.; Graf, G. A.; Smyth, S. S.; Morris, A. J., Mechanism of rapid elimination of lysophosphatidic acid and related lipids from the circulation of mice. *Journal of lipid research* **2013,** *54* (10), 2775-84.
158. Ramadass, P.; Meerarani, P.; Toborek, M.; Robertson, L. W.; Hennig, B., Dietary flavonoids modulate PCB-induced oxidative stress, CYP1A1 induction, and AhR-DNA binding activity in vascular endothelial cells. *Toxicological sciences : an official journal of the Society of Toxicology* **2003,** *76* (1), 212-9.
159. (a) Repetto, S.; Salani, B.; Maggi, D.; Cordera, R., Insulin and IGF-I phosphorylate eNOS in HUVECs by a caveolin-1 dependent mechanism. *Biochemical and biophysical research communications* **2005,** *337* (3), 849-52; (b) Zheng, Y.; Morris, A.; Sunkara, M.; Layne, J.; Toborek, M.; Hennig, B., Epigallocatechin-gallate stimulates NF-E2-related factor and heme oxygenase-1 via caveolin-1 displacement. *The Journal of nutritional biochemistry* **2012**.
160. Volonte, D.; Liu, Z.; Musille, P. M.; Stoppani, E.; Wakabayashi, N.; Di, Y. P.; Lisanti, M. P.; Kensler, T. W.; Galbiati, F., Inhibition of nuclear factor-erythroid 2-related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence. *Molecular biology of the cell* **2013,** *24* (12), 1852-62.

161. (a) Bourez, S.; Van den Daelen, C.; Le Lay, S.; Poupaert, J.; Larondelle, Y.; Thome, J. P.; Schneider, Y. J.; Dugail, I.; Debier, C., The dynamics of accumulation of PCBs in cultured adipocytes vary with the cell lipid content and the lipophilicity of the congener. *Toxicology letters* **2013,** *216* (1), 40-6; (b) Bourez, S.; Joly, A.; Covaci, A.; Remacle, C.; Larondelle, Y.; Schneider, Y. J.; Debier, C., Accumulation capacity of primary cultures of adipocytes for PCB-126: influence of cell differentiation stage and triglyceride levels. *Toxicology letters* **2012,** *214* (3), 243-50.
162. Eades, G.; Yang, M.; Yao, Y.; Zhang, Y.; Zhou, Q., miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *The Journal of biological chemistry* **2011,** *286* (47), 40725-33.
163. Pavlides, S.; Tsirigos, A.; Vera, I.; Flomenberg, N.; Frank, P. G.; Casimiro, M. C.; Wang, C.; Fortina, P.; Addya, S.; Pestell, R. G.; Martinez-Outschoorn, U. E.; Sotgia, F.; Lisanti, M. P., Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "reverse Warburg effect": a transcriptional informatics analysis with validation. *Cell cycle* **2010,** *9* (11), 2201-19.
164. Oka, N.; Yamamoto, M.; Schwencke, C.; Kawabe, J.; Ebina, T.; Ohno, S.; Couet, J.; Lisanti, M. P.; Ishikawa, Y., Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. *The Journal of biological chemistry* **1997,** *272* (52), 33416-21.
165. Das Evcimen, N.; King, G. L., The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacological research : the official journal of the Italian Pharmacological Society* **2007,** *55* (6), 498-510.
166. Feng, S.; Wang, Y.; Wang, X.; Wang, Z.; Cui, Y.; Liu, J.; Zhao, C.; Jin, M.; Zou, W., Caveolin-1 gene silencing promotes the activation of PI3K/AKT dependent on Eralpha36 and the transformation of MCF10ACE. *Science China. Life sciences* **2010,** *53* (5), 598-605.
167. Pi, J.; Bai, Y.; Reece, J. M.; Williams, J.; Liu, D.; Freeman, M. L.; Fahl, W. E.; Shugar, D.; Liu, J.; Qu, W.; Collins, S.; Waalkes, M. P., Molecular mechanism of human Nrf2 activation and degradation: role of sequential phosphorylation by protein kinase CK2. *Free radical biology & medicine* **2007,** *42* (12), 1797-806.
168. Naoum, J. J.; Zhang, S.; Woodside, K. J.; Song, W.; Guo, Q.; Belalcazar, L. M.; Hunter, G. C., Aortic eNOS expression and phosphorylation in Apo-E knockout mice: differing effects of rapamycin and simvastatin. *Surgery* **2004,** *136* (2), 323-8.
169. Meda, C.; Plank, C.; Mykhaylyk, O.; Schmidt, K.; Mayer, B., Effects of statins on nitric oxide/cGMP signaling in human umbilical vein endothelial cells. *Pharmacological reports : PR* **2010,** *62* (1), 100-12.
170. Weber, R.; Gaus, C.; Tysklind, M.; Johnston, P.; Forter, M.; Hollert, H.; Heinisch, E.; Holoubek, I.; Lloyd-Smith, M.; Masunaga, S.; Moccarelli, P.; Santillo, D.; Seike, N.; Symons, R.; Torres, J. P.; Verta, M.; Varbelow, G.; Vijgen, J.; Watson, A.; Costner, P.; Woelz, J.; Wycisk, P.; Zennegg, M., Dioxin- and POP-contaminated sites--contemporary and future relevance and challenges: overview on background, aims and scope of the series. *Environmental science and pollution research international* **2008,** *15* (5), 363-93.
171. Lind, P. M.; van Bavel, B.; Salihovic, S.; Lind, L., Circulating levels of persistent organic pollutants (POPs) and carotid atherosclerosis in the elderly. *Environmental health perspectives* **2012,** *120* (1), 38-43.
172. Valvi, D.; Mendez, M. A.; Martinez, D.; Grimalt, J. O.; Torrent, M.; Sunyer, J.; Vrijheid, M., Prenatal concentrations of polychlorinated biphenyls, DDE, and DDT and overweight in children: a prospective birth cohort study. *Environmental health perspectives* **2012,** *120* (3), 451-7.

173. Cave, M.; Appana, S.; Patel, M.; Falkner, K. C.; McClain, C. J.; Brock, G., Polychlorinated biphenyls, lead, and mercury are associated with liver disease in American adults: NHANES 2003-2004. *Environmental health perspectives* **2010,** *118* (12), 1735-42.
174. Hennig, B.; Toborek, M.; Bachas, L. G.; Suk, W. A., Emerging issues: nutritional awareness in environmental toxicology. *J Nutr Biochem* **2004,** *15* (4), 194-5.
175. Wahlang, B.; Falkner, K. C.; Gregory, B.; Ansert, D.; Young, D.; Conklin, D. J.; Bhatnagar, A.; McClain, C. J.; Cave, M., Polychlorinated biphenyl 153 is a diet-dependent obesogen that worsens nonalcoholic fatty liver disease in male C57BL6/J mice. *The Journal of nutritional biochemistry* **2013,** *24* (9), 1587-95.
176. De, S.; Ghosh, S.; Chatterjee, R.; Chen, Y. Q.; Moses, L.; Kesari, A.; Hoffman, E. P.; Dutta, S. K., PCB congener specific oxidative stress response by microarray analysis using human liver cell line. *Environment international* **2010,** *36* (8), 907-17.
177. Lai, I.; Chai, Y.; Simmons, D.; Luthe, G.; Coleman, M. C.; Spitz, D.; Haschek, W. M.; Ludewig, G.; Robertson, L. W., Acute toxicity of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in male Sprague-Dawley rats: effects on hepatic oxidative stress, glutathione and metals status. *Environment international* **2010,** *36* (8), 918-23.
178. Petriello, M. C.; Newsome, B.; Hennig, B., Influence of nutrition in PCB-induced vascular inflammation. *Environ Sci Pollut Res Int* **2013**.
179. (a) Myers, C. R., The effects of chromium(VI) on the thioredoxin system: implications for redox regulation. *Free radical biology & medicine* **2012,** *52* (10), 2091-107; (b) Mann, G. E.; Niehueser-Saran, J.; Watson, A.; Gao, L.; Ishii, T.; de Winter, P.; Siow, R. C., Nrf2/ARE regulated antioxidant gene expression in endothelial and smooth muscle cells in oxidative stress: implications for atherosclerosis and preeclampsia. *Sheng li xue bao : [Acta physiologica Sinica]* **2007,** *59* (2), 117-27.
180. Nordberg, J.; Arner, E. S., Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free radical biology & medicine* **2001,** *31* (11), 1287-312.
181. Savouret, J. F.; Berdeaux, A.; Casper, R. F., The aryl hydrocarbon receptor and its xenobiotic ligands: a fundamental trigger for cardiovascular diseases. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **2003,** *13* (2), 104-13.
182. Kopf, P. G.; Walker, M. K., 2,3,7,8-tetrachlorodibenzo-p-dioxin increases reactive oxygen species production in human endothelial cells via induction of cytochrome P4501A1. *Toxicology and applied pharmacology* **2010,** *245* (1), 91-9.
183. Lee, J. M.; Chan, K.; Kan, Y. W.; Johnson, J. A., Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (26), 9751-6.
184. Rangasamy, T.; Cho, C. Y.; Thimmulappa, R. K.; Zhen, L.; Srisuma, S. S.; Kensler, T. W.; Yamamoto, M.; Petrache, I.; Tuder, R. M.; Biswal, S., Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* **2004,** *114* (9), 1248-59.
185. Zhen, M. C.; Wang, Q.; Huang, X. H.; Cao, L. Q.; Chen, X. L.; Sun, K.; Liu, Y. J.; Li, W.; Zhang, L. J., Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride-induced hepatic fibrosis. *The Journal of nutritional biochemistry* **2007,** *18* (12), 795-805.
186. Bruno, R. S.; Dugan, C. E.; Smyth, J. A.; DiNatale, D. A.; Koo, S. I., Green tea extract protects leptin-deficient, spontaneously obese mice from hepatic steatosis and injury(1,2). *Journal of Nutrition* **2008,** *138* (2), 323-331.
187. Montuschi, P.; Barnes, P.; Roberts, L. J., Insights into oxidative stress: The isoprostanes. *Current Medicinal Chemistry* **2007,** *14* (6), 703-717.

188. Dorjgochoo, T.; Gao, Y. T.; Chow, W. H.; Shu, X. O.; Yang, G.; Cai, Q.; Rothman, N.; Cai, H.; Li, H.; Deng, X.; Franke, A.; Roberts, L. J.; Milne, G.; Zheng, W.; Dai, Q., Major metabolite of F2-isoprostane in urine may be a more sensitive biomarker of oxidative stress than isoprostane itself. *Am J Clin Nutr* **2012,** *96* (2), 405-14.
189. Hennig, B.; Ettinger, A. S.; Jandacek, R. J.; Koo, S.; McClain, C.; Seifried, H.; Silverstone, A.; Watkins, B.; Suk, W. A., Using nutrition for intervention and prevention against environmental chemical toxicity and associated diseases. *Environmental health perspectives* **2007,** *115* (4), 493-5.
190. Cave, M.; Deaciuc, I.; Mendez, C.; Song, Z.; Joshi-Barve, S.; Barve, S.; McClain, C., Nonalcoholic fatty liver disease: predisposing factors and the role of nutrition. *The Journal of nutritional biochemistry* **2007,** *18* (3), 184-95.
191. (a) Chung, M. Y.; Park, H. J.; Manautou, J. E.; Koo, S. I.; Bruno, R. S., Green tea extract protects against nonalcoholic steatohepatitis in ob/ob mice by decreasing oxidative and nitrative stress responses induced by proinflammatory enzymes. *The Journal of nutritional biochemistry* **2012,** *23* (4), 361-7; (b) Park, H. J.; DiNatale, D. A.; Chung, M. Y.; Park, Y. K.; Lee, J. Y.; Koo, S. I.; O'Connor, M.; Manautou, J. E.; Bruno, R. S., Green tea extract attenuates hepatic steatosis by decreasing adipose lipogenesis and enhancing hepatic antioxidant defenses in ob/ob mice. *J Nutr Biochem* **2011,** *22* (4), 393-400.
192. (a) Sipos, E.; Chen, L.; Andras, I. E.; Wrobel, J.; Zhang, B.; Pu, H.; Park, M.; Eum, S. Y.; Toborek, M., Proinflammatory adhesion molecules facilitate polychlorinated biphenyl-mediated enhancement of brain metastasis formation. *Toxicological sciences : an official journal of the Society of Toxicology* **2012,** *126* (2), 362-71; (b) Seelbach, M.; Chen, L.; Powell, A.; Choi, Y. J.; Zhang, B.; Hennig, B.; Toborek, M., Polychlorinated biphenyls disrupt blood-brain barrier integrity and promote brain metastasis formation. *Environ Health Perspect* **2010,** *118* (4), 479-84.
193. Cabrera, C.; Artacho, R.; Gimenez, R., Beneficial effects of green tea--a review. *Journal of the American College of Nutrition* **2006,** *25* (2), 79-99.
194. (a) Schlezinger, J. J.; Stegeman, J. J., Induction and suppression of cytochrome P450 1A by 3,3',4,4',5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (Stenotomus chrysops). *Aquatic toxicology* **2001,** *52* (2), 101-15; (b) Twaroski, T. P.; O'Brien, M. L.; Robertson, L. W., Effects of selected polychlorinated biphenyl (PCB) congeners on hepatic glutathione, glutathione-related enzymes, and selenium status: implications for oxidative stress. *Biochemical pharmacology* **2001,** *62* (3), 273-81; (c) Katynski, A. L.; Vijayan, M. M.; Kennedy, S. W.; Moon, T. W., 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) impacts hepatic lipid peroxidation, membrane fluidity and beta-adrenoceptor kinetics in chick embryos. *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* **2004,** *137* (1), 81-93; (d) Hassoun, E. A.; Li, F.; Abushaban, A.; Stohs, S. J., The relative abilities of TCDD and its congeners to induce oxidative stress in the hepatic and brain tissues of rats after subchronic exposure. *Toxicology* **2000,** *145* (2-3), 103-13.
195. Suzuki, T.; Hara, H., Role of flavonoids in intestinal tight junction regulation. *The Journal of nutritional biochemistry* **2011,** *22* (5), 401-8.
196. Frei, B.; Higdon, J. V., Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J Nutr* **2003,** *133* (10), 3275S-84S.
197. (a) Na, H. K.; Surh, Y. J., Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food Chem Toxicol* **2008,** *46* (4), 1271-8; (b) Scapagnini, G.; Vasto, S.; Abraham, N. G.; Caruso, C.; Zella, D.; Fabio, G., Modulation of Nrf2/ARE pathway by food polyphenols: a nutritional neuroprotective strategy for cognitive and neurodegenerative disorders. *Molecular neurobiology* **2011,** *44* (2), 192-201.

198. Lorenz, M.; Urban, J.; Engelhardt, U.; Baumann, G.; Stangl, K.; Stangl, V., Green and black tea are equally potent stimuli of NO production and vasodilation: new insights into tea ingredients involved. *Basic research in cardiology* **2009,** *104* (1), 100-10.
199. (a) Skrzydlewska, E.; Ostrowska, J.; Farbiszewski, R.; Michalak, K., Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain. *Phytomedicine : international journal of phytotherapy and phytopharmacology* **2002,** *9* (3), 232-8; (b) Guleria, R. S.; Jain, A.; Tiwari, V.; Misra, M. K., Protective effect of green tea extract against the erythrocytic oxidative stress injury during mycobacterium tuberculosis infection in mice. *Molecular and cellular biochemistry* **2002,** *236* (1-2), 173-81.
200. Williams, S. N.; Shih, H.; Guenette, D. K.; Brackney, W.; Denison, M. S.; Pickwell, G. V.; Quattrochi, L. C., Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A gene expression. *Chemico-biological interactions* **2000,** *128* (3), 211-29.
201. (a) Pae, M.; Ren, Z.; Meydani, M.; Shang, F.; Smith, D.; Meydani, S. N.; Wu, D., Dietary supplementation with high dose of epigallocatechin-3-gallate promotes inflammatory response in mice. *The Journal of nutritional biochemistry* **2012,** *23* (6), 526-31; (b) Chan, P. C.; Ramot, Y.; Malarkey, D. E.; Blackshear, P.; Kissling, G. E.; Travlos, G.; Nyska, A., Fourteen-week toxicity study of green tea extract in rats and mice. *Toxicologic pathology* **2010,** *38* (7), 1070-84.
202. Birringer, M., Hormetics: dietary triggers of an adaptive stress response. *Pharmaceutical research* **2011,** *28* (11), 2680-94.
203. Lazic, M.; Inzaugarat, M. E.; Povero, D.; Zhao, I. C.; Chen, M.; Nalbandian, M.; Miller, Y. I.; Chernavsky, A. C.; Feldstein, A. E.; Sears, D. D., Reduced dietary omega-6 to omega-3 fatty acid ratio and 12/15-lipoxygenase deficiency are protective against chronic high fat diet-induced steatohepatitis. *PloS one* **2014,** *9* (9), e107658.
204. Moran, J. H.; Mitchell, L. A.; Bradbury, J. A.; Qu, W.; Zeldin, D. C.; Schnellmann, R. G.; Grant, D. F., Analysis of the cytotoxic properties of linoleic acid metabolites produced by renal and hepatic P450s. *Toxicology and applied pharmacology* **2000,** *168* (3), 268-79.
205. Slim, R.; Hammock, B. D.; Toborek, M.; Robertson, L. W.; Newman, J. W.; Morisseau, C. H.; Watkins, B. A.; Saraswathi, V.; Hennig, B., The role of methyl-linoleic acid epoxide and diol metabolites in the amplified toxicity of linoleic acid and polychlorinated biphenyls to vascular endothelial cells. *Toxicology and applied pharmacology* **2001,** *171* (3), 184-93.
206. Shin, E.; Yeo, E.; Lim, J.; Chang, Y. H.; Park, H.; Shim, E.; Chung, H.; Hwang, H. J.; Chun, J.; Hwang, J., Nitrooleate mediates nitric oxide synthase activation in endothelial cells. *Lipids* **2014,** *49* (5), 457-66.
207. Hennig, B.; Toborek, M.; McClain, C. J.; Diana, J. N., Nutritional implications in vascular endothelial cell metabolism. *Journal of the American College of Nutrition* **1996,** *15* (4), 345-58.
208. (a) Reddy, A. T.; Lakshmi, S. P.; Reddy, R. C., The Nitrated Fatty Acid 10-Nitro-oleate Diminishes Severity of LPS-Induced Acute Lung Injury in Mice. *PPAR research* **2012,** *2012*, 617063; (b) Zhang, J.; Villacorta, L.; Chang, L.; Fan, Z.; Hamblin, M.; Zhu, T.; Chen, C. S.; Cole, M. P.; Schopfer, F. J.; Deng, C. X.; Garcia-Barrio, M. T.; Feng, Y. H.; Freeman, B. A.; Chen, Y. E., Nitro-oleic acid inhibits angiotensin II-induced hypertension. *Circulation research* **2010,** *107* (4), 540-8; (c) Wang, H.; Liu, H.; Jia, Z.; Olsen, C.; Litwin, S.; Guan, G.; Yang, T., Nitro-oleic acid protects against endotoxin-induced endotoxemia and multiorgan injury in mice. *American journal of physiology. Renal physiology* **2010,** *298* (3), F754-62.

209. Nannelli, A.; Rossignolo, F.; Tolando, R.; Rossato, P.; Longo, V.; Gervasi, P. G., Effect of beta-naphthoflavone on AhR-regulated genes (CYP1A1, 1A2, 1B1, 2S1, Nrf2, and GST) and antioxidant enzymes in various brain regions of pig. *Toxicology* **2009,** *265* (3), 69-79.
210. Villard, P. H.; Barlesi, F.; Armand, M.; Dao, T. M.; Pascussi, J. M.; Fouchier, F.; Champion, S.; Dufour, C.; Ginies, C.; Khalil, A.; Amiot, M. J.; Barra, Y.; Seree, E., CYP1A1 induction in the colon by serum: involvement of the PPARalpha pathway and evidence for a new specific human PPREalpha site. *PloS one* **2011,** *6* (1), e14629.
211. Viswanathan, S.; Hammock, B. D.; Newman, J. W.; Meerarani, P.; Toborek, M.; Hennig, B., Involvement of CYP 2C9 in mediating the proinflammatory effects of linoleic acid in vascular endothelial cells. *Journal of the American College of Nutrition* **2003,** *22* (6), 502-10.
212. Spiteller, G., Linoleic acid peroxidation--the dominant lipid peroxidation process in low density lipoprotein--and its relationship to chronic diseases. *Chemistry and physics of lipids* **1998,** *95* (2), 105-62.
213. Wang, L.; Lim, E. J.; Toborek, M.; Hennig, B., The role of fatty acids and caveolin-1 in tumor necrosis factor alpha-induced endothelial cell activation. *Metabolism: clinical and experimental* **2008,** *57* (10), 1328-39.
214. Zheng, Y.; Lim, E. J.; Wang, L.; Smart, E. J.; Toborek, M.; Hennig, B., Role of caveolin-1 in EGCG-mediated protection against linoleic-acid-induced endothelial cell activation. *The Journal of nutritional biochemistry* **2009,** *20* (3), 202-9.
215. Massaro, M.; Scoditti, E.; Carluccio, M. A.; De Caterina, R., Nutraceuticals and prevention of atherosclerosis: focus on omega-3 polyunsaturated fatty acids and Mediterranean diet polyphenols. *Cardiovasc Ther* **2010,** *28* (4), e13-9.
216. Moertl, D.; Hammer, A.; Steiner, S.; Hutuleac, R.; Vonbank, K.; Berger, R., Dose-dependent effects of omega-3-polyunsaturated fatty acids on systolic left ventricular function, endothelial function, and markers of inflammation in chronic heart failure of nonischemic origin: a double-blind, placebo-controlled, 3-arm study. *Am Heart J* **2011,** *161* (5), 915 e1-9.
217. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L., Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **2004,** *79* (5), 727-47.
218. Kris-Etherton, P. M.; Lefevre, M.; Beecher, G. R.; Gross, M. D.; Keen, C. L.; Etherton, T. D., Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annu Rev Nutr* **2004,** *24*, 511-38.
219. (a) D'Archivio, M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R., Polyphenols, dietary sources and bioavailability. *Ann Ist Super Sanita* **2007,** *43* (4), 348-61; (b) Aron, P. M.; Kennedy, J. A., Flavan-3-ols: nature, occurrence and biological activity. *Molecular nutrition & food research* **2008,** *52* (1), 79-104; (c) Gonzalez-Gallego, J.; Garcia-Mediavilla, M. V.; Sanchez-Campos, S.; Tunon, M. J., Fruit polyphenols, immunity and inflammation. *The British journal of nutrition* **2010,** *104 Suppl 3*, S15-27.
220. (a) Casper, R. F.; Quesne, M.; Rogers, I. M.; Shirota, T.; Jolivet, A.; Milgrom, E.; Savouret, J. F., Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. *Mol Pharmacol* **1999,** *56* (4), 784-90; (b) Singhal, R.; Badger, T. M.; Ronis, M. J., Reduction in 7,12-dimethylbenz[a]anthracene-induced hepatic cytochrome-P450 1A1 expression following soy consumption in female rats is mediated by degradation of the aryl hydrocarbon receptor. *J Nutr* **2007,** *137* (1), 19-24; (c) Palermo, C. M.; Westlake, C. A.; Gasiewicz, T. A., Epigallocatechin gallate inhibits aryl hydrocarbon receptor gene transcription through an indirect mechanism involving binding to a 90 kDa heat shock protein. *Biochemistry* **2005,** *44* (13), 5041-52.

221. Majkova, Z.; Layne, J.; Sunkara, M.; Morris, A. J.; Toborek, M.; Hennig, B., Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls. *Toxicol Appl Pharmacol 251* (1), 41-9.
222. Hennig, B.; Ormsbee, L.; McClain, C. J.; Watkins, B. A.; Blumberg, B.; Bachas, L. G.; Sanderson, W.; Thompson, C.; Suk, W. A., Nutrition Can Modulate the Toxicity of Environmental Pollutants: Implications in Risk Assessment and Human Health. *Environ Health Perspect* **2012**.
223. Fu, Y.; Moore, X. L.; Lee, M. K.; Fernandez-Rojo, M. A.; Parat, M. O.; Parton, R. G.; Meikle, P. J.; Sviridov, D.; Chin-Dusting, J. P., Caveolin-1 plays a critical role in the differentiation of monocytes into macrophages. *Arteriosclerosis, thrombosis, and vascular biology* **2012,** *32* (9), e117-25.
224. Jensen, A., Background levels in humans In *Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products* Kimbrough, R.; Jensen, A., Eds. Elsevier: New York, 1989.
225. (a) Sandanger, T. M.; Brustad, M.; Odland, J. O.; Doudarev, A. A.; Miretsky, G. I.; Chaschin, V.; Burkow, I. C.; Lund, E., Human plasma levels of POPs, and diet among native people from Uelen, Chukotka. *J Environ Monit* **2003,** *5* (4), 689-96; (b) Henriquez-Hernandez, L. A.; Luzardo, O. P.; Almeida-Gonzalez, M.; Alvarez-Leon, E. E.; Serra-Majem, L.; Zumbado, M.; Boada, L. D., Background levels of polychlorinated biphenyls in the population of the Canary Islands (Spain). *Environ Res 111* (1), 10-6.
226. Tsikas, D.; Zoerner, A.; Mitschke, A.; Homsi, Y.; Gutzki, F. M.; Jordan, J., Specific GC-MS/MS stable-isotope dilution methodology for free 9- and 10-nitro-oleic acid in human plasma challenges previous LC-MS/MS reports. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2009,** *877* (26), 2895-908.
227. Mineo, C.; Shaul, P. W., Regulation of eNOS in caveolae. *Advances in experimental medicine and biology* **2012,** *729*, 51-62.
228. Murphy, M. O.; Petriello, M. C.; Han, S. G.; Sunkara, M.; Morris, A. J.; Esser, K.; Hennig, B., Exercise protects against PCB-induced inflammation and associated cardiovascular risk factors. *Environmental science and pollution research international* **2015**.

Vita

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Education

B.S. in Biology and Environmental Science from Muhlenberg College, Allentown, PA, 2010

Professional positions held

Research Assistant, Muhlenberg College, Allentown, PA, 2007-2010
Peer tutor, Muhlenberg College, Allentown, PA, 2007-2010

Scholastic and professional honors

1. CEECHE 2014 Meeting Travel Support Award, Romania, 05/2014
2. SOT Annual Meeting Travel Support Award, Phoenix, AZ, 12/2013
3. SETAC Travel Award, Nashville, TN, 11/2013
4. Superfund Annual Meeting Plenary Speaker Travel Award, Baton Rouge, LA, 10/2013
5. American Heart Association Great Rivers Affiliate Predoctoral Fellowship, University of Kentucky, 07/2013-06/2015
6. NIEHS T32 Training Grant Recipient, University of Kentucky, 06/2011-06/2013
7. Dean's Grant Scholarship, Muhlenberg College, Allentown, PA, 03/2009
8. Merck Scholar Summer Research Award, Muhlenberg College, Allentown, PA, 05/2008
9. Presidential Scholarship, Muhlenberg College, Allentown, PA, 08/2008

10. Eagle Scout, Troop 105, Schwenksville, PA, 08/2006

Professional publications

1. Murphy MO, **Petriello MC**, Han SG, Sunkara M, Morris AJ, Esser K, Hennig B: Exercise protects against PCB-induced inflammation and associated cardiovascular risk factors. *Environmental science and pollution research*. 2015 Jan 15. Epub ahead of print. PMID: 25586614

2. **Petriello MC**, Han SG, Newsome B, Hennig B: PCB 126 toxicity is modulated by cross-talk between caveolae and Nrf2 signaling. 2014 Jun 1;277(2):192-9. doi: 10.1016/j.taap.2014.03.018. PMID: 24709675.

3. **Petriello MC**, Newsome B, Dziubla TD, Hilt JZ, D Bhattacharyya, Hennig B: Modulation of persistent organic pollutant toxicity through nutritional intervention: emerging opportunities in biomedicine and environmental remediation. Science *of the total environment*. 2014. 21(10); 6410-6418. PMID: 24530186

4. **Petriello MC**, Newsome B, Han SG, Murphy M, Eske K, Sunkara M, Morris A, Hennig B: Green tea diet decreases PCB 126-induced oxidative stress in mice by upregulating antioxidant enzymes. *Journal of Nutritional Biochemistry.* 2014 Feb;25(2):126-35. doi: 10.1016/j.jnutbio.2013.10.003. Epub 2013 Nov 6. PMID: 24378064

5. **Petriello MC**, Newsome B, Hennig B: Influence of nutrition in PCB-induced vascular inflammation. *Environmental Science Pollution Research.* 2014 May;21(10):6410-8.. 10.1007/s11356-013-1549-5. Epub 2013 Feb 17 PMID: 23417440

6. Kelsey JW, Slizovskiy IB, **Petriello MC**, Butler KL.: Influence of plant-earthworm interactions on SOM chemistry and *p,p'*-DDE bioaccumulation. *Chemosphere.* 2011 May;83(7):897-902. doi: 10.1016/j.chemosphere.2011.02.056. Epub 2011 Mar 21. PMID: 21421253